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Clinical-size Dextran by Direct Fermentation (with NRRL B–1351 *Streptococcus sp.*)*

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Summary. Dextran, a polymer of glucose, has been widely used as a blood-plasma volume expander. In whole culture fermentations most dextran-producing organisms elaborate dextrans that have molecular weights of many millions and must be depolymerized and fractionated to obtain a product suitable for clinical use. An exception however is *Streptococcus sp.* (Strain DS–50) NRRL B–1351. In the work described here, this organism is used to produce clinical-size dextran in high yields, both on a laboratory- and a pilot-plant scale. With this process, fractionation is required but depolymerization is not. Fermentations of media containing 15 per cent sucrose are complete in less than 48 hours with 0·2 per cent inoculum. Yields of native dextran and clinical-size dextran by methanol precipitation are 68 and 43 per cent of the theory, respectively. The structurally heterogeneous dextran formed by NRRL B–1351 has a higher viscosity for a given molecular weight than does NRRL B–512 dextran. The viscosity–molecular weight relationship for the former may be expressed by the equation $[\eta] = 6{\cdot}51 \times 10^{-4} M_w^{0{\cdot}554}$. The high-molecular weight fraction had approximately 75 per cent of 1,6-like linkages, as compared with 87 per cent for the clinical fraction. All fractions had a lower ratio of 1,6 to non-1,6 linkages than did NRRL B–512 dextrans.

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Introduction

Dextrans formed in whole culture bacterial fermentation[1] or by cell-free enzymes from *Leuconostoc mesenteroides* NRRL B–512F[2,3] generally have molecular weights ranging from several million to several hundred million and must be depolymerized and fractionated to obtain products suitable for clinical use.[4–7] Workers in this laboratory[8] and others[9] have previously described processes for the production of native dextran and for its reduction to clinical-size by acid hydrolysis. A second phase of the work here showed that clinical-size dextran could be made by a controlled enzymatic synthesis using oligosaccharides or low-molecular-weight dextran as glucosyl acceptors or primers.[3, 10] However, use of a primer dextran necessitated a hydrolysis step.

Hehre has shown that certain nonhaemolytic streptococci (*Streptococcus* var. DS) convert sucrose to dextran and that strain DS–50 synthesizes a product of relatively low molecular weight.[11] Moreover, a major part of the dextran lies in the molecular weight range desired for clinical use.[12, 13]

This paper describes conditions for the production in whole culture fermentations with NRRL B–1351 *Streptococcus sp.* (Strain DS–50) of clinical-size dextran in high yield. With this process fractionation is required but depolymerization is not. Certain physical and chemical properties of this dextran are also discussed.

Laboratory Investigations

Unlike most other dextran-synthesizing organisms, NRRL B–1351 was difficult to maintain in stock culture on standard media not containing blood or other body fluids. This difficulty was overcome by incorporating 1 per cent tomato juice in the maintenance medium which contained 1 per cent yeast hydrolyzate, 3 per cent dipotassium phosphate, and 10 per cent sucrose and was adjusted to a pH of 7·4. Stock cultures were kept in liquid and were transferred three times weekly. Optimal inoculum size, as well as the nutrient composition of the inoculum and fermentation media, temperature, and pH of fermentation were determined during laboratory investigations. The results shown in Fig. 1 demonstrate that a low percentage, 0·1 to 0·2 per

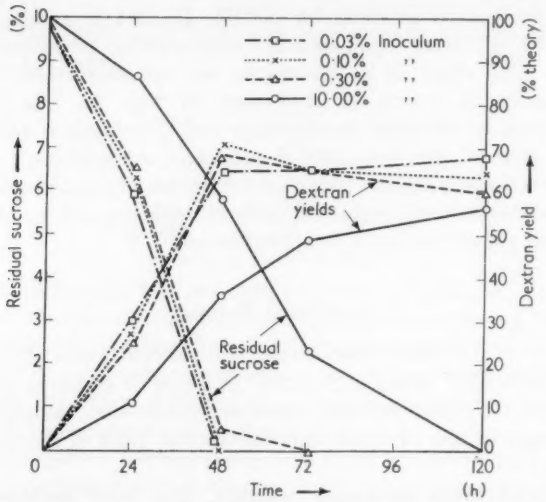

Fig. 1. A small percentage of inoculum gave better yields than a large percentage of inoculum, and shortened the fermentation time

cent, of inoculum gave good dextran yields in fermentations of less than 48 h duration. With 5 to 10 per cent inoculum, usually employed for other dextran fermentations, 5 to 7 days were needed to metabolize the sucrose in the medium.

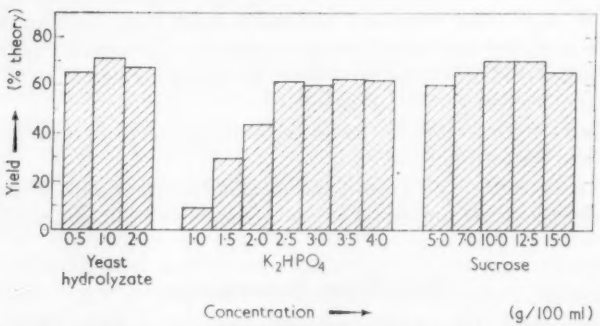

Fig. 2. The yield of dextran is affected by the concentration of the components in the medium

The production medium for NRRL B–1351 is a simple one, composed of yeast hydrolyzate, dipotassium phosphate, and sucrose. The effect of variations in the concentrations of these components on yields is illustrated in Fig. 2. The optimal concentrations of yeast hydrolyzate and dipotassium phosphate chosen were 1 per cent and 3 per cent, respectively; sucrose concentrations as high as 15 per cent could be fermented efficiently. Fermentations were conducted without aeration and agitation at an initial pH of 7·4 and a temperature of 97°F.

Analytical Methods

Yields of dextran were based on concentration determined polarimetrically, using $[\alpha]_D^{25°} + 200°$ in water or aqueous methanol solutions. Unless reported otherwise, yields were calculated as a percentage of the 47·4 per cent theoretical yield of dextran from sucrose.

Weight-average molecular weights, M_w, were determined by the light-scattering method using a Brice–Speiser light-scattering photometer.[14] The complete procedure employed during these studies for determining molecular weights by light scattering has been described by Senti *et al.*[15]

Number-average molecular weights, M_n, were calculated by measurement of the fructose of the dextran fractions[16, 17] on the assumption that there is one fructose residue per molecule.

Fructose was determined by the alcoholic–anthrone method of Wise *et al.*,[17] and viscosity determinations were conducted at 25°C in jacketed capillary-flow viscosimeters. Inherent viscosities were calculated from the flow time for solutions which exhibited a relative viscosity of 1·10–1·50.[6, 18]

Sodium metaperiodate oxidation analyses were determined as described previously.[19]

Dextran solubility curves were obtained by methanol precipitation and provided a measure of the molecular weight distribution.[3]

Pilot-Plant Investigation

Equipment. Pilot-plant fermentations were conducted in three fermentors of 600-, 300-, and 60-gal capacities constructed from

types 316, 302, and 304 stainless steel, respectively. Their dimensions were:

Size, gal	Height, in.	Diameter, in.
600	90	48
300	156	24
60	48	22

Each fermentor had an agitator, was jacketed, and equipped with automatic controls to maintain temperature during fermentation within 1·0° of the desired temperature (97°F).

Either of two cooker sterilizers—one made of type 304 stainless steel and the other of Herculoy, a copper–bronze alloy—was used for continuous sterilization of the medium.

Centrifugation for removing cells from the fermented medium was accomplished with a No. 16 Sharples Supercentrifuge, which at its normal rotational speed of 15,000 rev/min developed a centrifugal force of 13,200 times the force of gravity.

Precipitation and fractionation operations were carried out in type 304 stainless-steel tanks of 55-gal capacity. They were equipped with removable stainless-steel coils so that a temperature of 77°F ± 1·0° could be maintained during fractionation. Agitation was accomplished with propeller-type agitators.

A stainless-steel laboratory-size spray dryer was used to obtain a dry product. The desiccator had a diameter of 4 ft and a height of 10 ft 6 in.

Process. The major process steps were: production of inoculum; fermentation; purification to remove bacterial cells and other impurities; fractionation to obtain clinical-size dextran; and dehydration. A flowsheet of this process is shown in Fig. 3.

Production of inoculum. All inocula were cultured at 97°F without aeration or agitation. A two-step process was adopted for the production of inocula. In step 1, a 24-h stock culture was used to seed a medium containing 10 per cent commercial sucrose, 1 per cent yeast hydrolyzate, 3 per cent dipotassium phosphate, and 1 per cent tomato juice (v/v) which had been adjusted to pH 7·4. After incubation for 22–24 h, the process was repeated in step 2. After 18–22 h, 0·1 to 0·2 per cent by

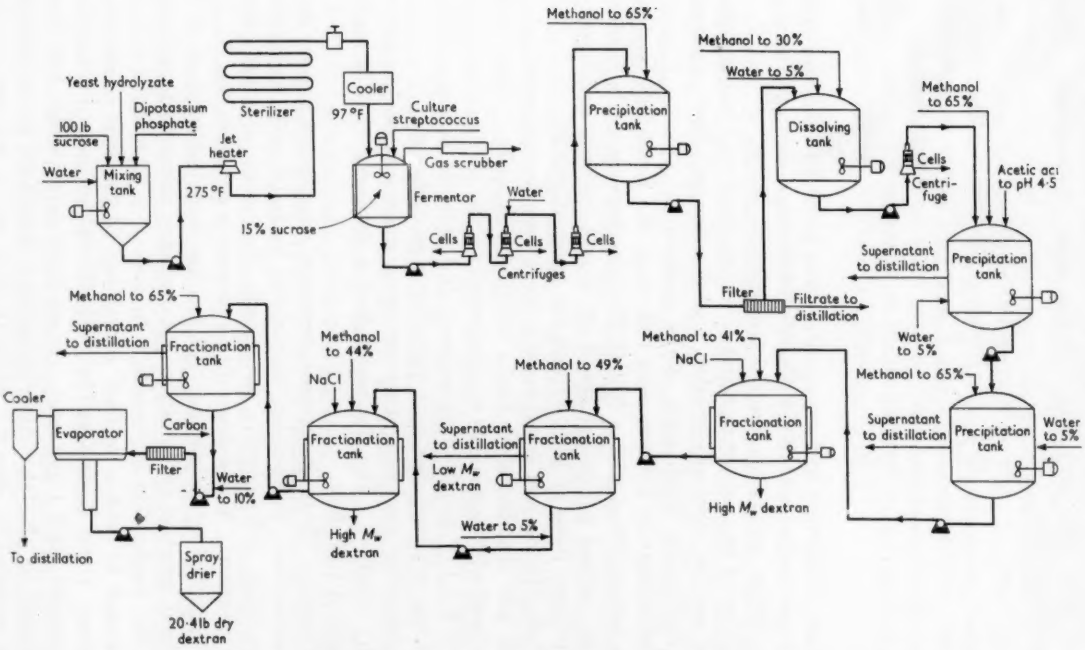

Fig. 3. A powdered clinical-size dextran was produced by a proper sequence of processing techniques

volume of inoculum was transferred aseptically to the fermentor. As shown in Table I, the use of inoculum having a pH between 4·6 and 5·2 permitted completion of fermentations in 48 h.

Table I. Fermentation time as affected by pH of inoculum

Inoculum pH	Fermentation time, h	Fermentation yield, % theory
7·0	70	59·3
6·8	68	67·5
5·2	46	67·2
5·0	48	65·5
4·9	45	71·4
4·8	46	66·2
4·7	45	72·3
4·6	45	70·1

Medium and sterilization. The composition of the medium used in the first pilot-plant runs was 10 per cent commercial sucrose, 3 per cent dipotassium phosphate, and 1 per cent yeast hydrolyzate. In later runs the sucrose was increased to 15 per cent. As shown by the results (Table II and Fig. 4) the use of 15 per cent sucrose in pilot-plant fermentations did not adversely affect the dextran yield or increase the fermentation time. Because an occasional batch of the yeast hydrolyzate did not support good growth, test samples were run in laboratory-scale fermentations before pilot-plant use.

Media were continuously sterilized at a pH of 7·4 for 4 min at 275°F by two different methods. In the first the ingredients were dissolved in separate vessels; in the second all ingredients were mixed together and dissolved in the same vessel. Since there was no difference in the sterility of the medium or yield of dextran obtained, the second method, requiring less equipment, was adopted as the standard procedure.

Copper content of the media before sterilization was about 0·2 ppm.; after sterilization in the Herculoy cooker it ranged from 70 to greater than 100 ppm. When the copper content was greater than 80 ppm., the organism could not initiate growth. At the lower copper value of 70 ppm. fermentations were very slow.

Table II. Yield and characterization of native dextran produced in three fermentors. (Nos. 1, 2 and 3 are the serial precipitations)

Fermentor capacity, gal	Medium volume, gal	No. of runs	Sucrose, %	Average yield[a] 0–65% ppt.			Average nitrogen 0–65% ppt.			Average ash 0–65% ppt.		
				No. 1	No. 2	No. 3[b]	No. 1	No. 2	No. 3	No. 1	No. 2	No. 3
60	40	1	10	67·8	66·5	66·5	0·55	0·25	0·20	19·1	2·9	0·6
60	40	6	15	68·6	64·1	62·2	0·25	0·11	0·07	13·5	2·1	0·3
300	120	2	10	58·7	57·0	55·9	0·49	0·21	0·18	16·8	2·9	0·5
600	300	1	15	70·1	66·5	63·2	0·67	0·17	0·14	10·6	1·8	0·3

[a] Per cent of theory.
[b] After three precipitations at 65 per cent methanol concentration.

Since media from the stainless-steel sterilizer had less than 6 ppm. copper, this cooker was used to prepare sterile media for all subsequent production runs.

Fermentation. All equipment was steam-sterilized at 250–260°F for 2 h before use. The medium was pumped at a constant rate to a steam jet heater where it was mixed with sufficient steam to raise the temperature to 275°F instantaneously. After 4-min retention, the medium, in its continuous passage to the fermentor, was cooled in a spiral heat exchanger to the required fermentation temperature. After inoculation of the fermentor with 0·1–0·2 per

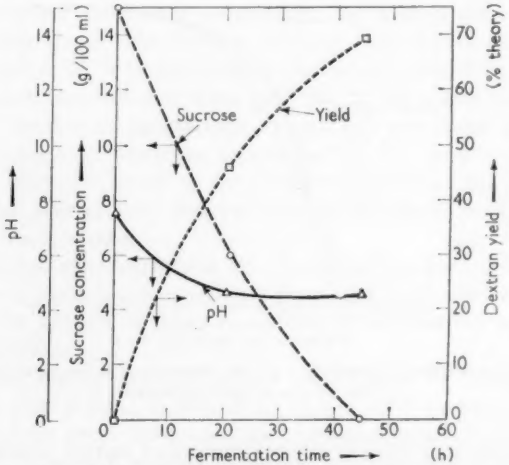

Fig. 4. Good yields of dextran were obtained with 15 per cent sucrose without an increase in fermentation time

cent of its volume with a liquid culture of NRRL B–1351, the fermentation was conducted without aeration or agitation and the temperature was controlled at 97°F.

The progress of the fermentation was followed by determining the concentration of sucrose, the pH, and the yield of dextran. Changes in pH, sucrose concentration, and dextran concentration which occur during a typical fermentation are shown in Fig. 4.

When disappearance of the sucrose indicated that the fermentation was complete (48 h, or less), the fermented material was

heated to 212°F as a precautionary measure to kill the bacterial cells, and then cooled prior to dextran recovery.

Separation and purification of the native dextran. Substantially complete removal of cells was accomplished by three centrifugations of fermentation medium originally containing 10 per cent sucrose; medium originally containing 15 per cent sucrose, although not very viscous, was diluted after two centrifugations with one-half volume of water and then centrifuged for the third time. To the essentially cell-free centrifugate, methanol was added with agitation to a concentration of 65 per cent by volume.

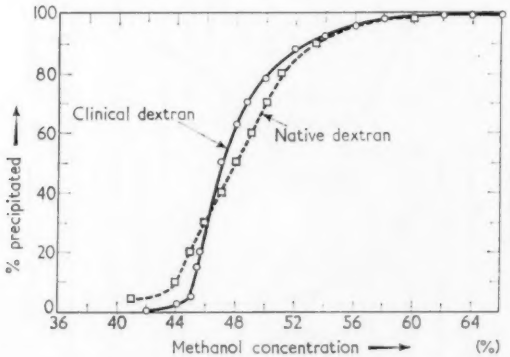

Fig. 5. Methanol precipitation showed the presence of a bi-modal distribution of the native dextran produced

The mixture was filtered immediately and native dextran, as a granular product which contained 11–20 per cent ash, was recovered in yields of 60–72 per cent of the theoretical yield.

To remove from the first precipitate salts which adversely affect the precipitation characteristics of dextran during fractionation and are not desirable in the clinical product, the native dextran was precipitated a second and third time to decrease the ash content to below 1 per cent. This decrease was accomplished by dissolving the dextran in water to 5 per cent concentration, as determined by optical rotation measurements, and then adding methanol with agitation to 30 per cent concentration (v/v). Residual bacterial cells were removed by supercentrifugation and

the slightly cloudy centrifugate was adjusted to pH 4·5 with acetic acid. Additional methanol was added to 65 per cent concentration to precipitate the native dextran for the second time. The product, in the form of a sticky gum, was recovered in yields of 60 to 67 per cent by decanting the supernatant. It was again dissolved in water to 5 per cent concentration and re-precipitated by adding methanol to 65 per cent concentration. The purified native dextran, obtained in yields of 55 to 66 per cent after the third precipitation, was a sticky gum with an ash content of less than 1 per cent. Yields and characterization of the native dextran obtained and its alcohol precipitation characteristics are shown in Table II and Fig. 5, respectively. Methanol was recovered from each supernatant by distillation.

Preparation of clinical-size dextran. All fractionations were conducted at 77°F by addition of methanol to solutions containing 5 per cent dextran and were held for 20–24 h to permit the insoluble dextran to precipitate. Since salt was found to promote more rapid and complete separation of the phases, 1 per cent sodium chloride (based on the weight of dextran) was added each time the precipitated dextran was made up in water solution during fractionation.

Methanol concentrations for fractionation are reported on a volume–volume basis calculated from methanol addition without regard for volume changes on mixing or for alcohol carried over in precipitates.

Purified native dextran in water solution at 77°F was fractionated by first adding methanol to 41 per cent concentration; the fraction that precipitated at this methanol concentration had an average molecular weight of several millions and was removed. Methanol was then added to the supernatant until its concentration was increased to 49 per cent. The crude clinical fraction, which precipitated as a fluid gum, was recovered by decanting the supernatant. A product obtained by this procedure in yields of 43–45 per cent of theory had an average molecular weight of 75,000, the midpoint of the specified range for clinical material. A parallel procedure which utilized methanol concentration limits of 42–50 per cent, gave a crude clinical fraction in yields of 35–38 per cent of theory, with an average molecular weight of about 60,000. The latter was considered too close to the lower limit of

Table III. Yields and molecular weights of dextran fractions obtained at selected methanol concentrations from purified native dextran

Run no.	0–42% Yield			42–50% Yield			50–65% Yield			>65% Yield
	native,[a] %	theory,[b] %	M_w	native,[a] %	theory,[b] %	M_w	native,[a] %	theory,[b] %	M_w	native,[a] %
3[c]	33·9	19·1	$22{\cdot}9 \times 10^6$	51·5	29·0	92,000	—	—	—	—
6[d]	18·2	10·1	103×10^6	66·9	37·1	113,000	10·9	6·1	—	3·9
7[d]	30·3	20·2	—	53·0	35·2	62,000	5·3	3·5	29,000	8·0
8[e]	22·2	13·3	$25{\cdot}3 \times 10^6$	63·6	38·2	66,000	10·0	6·0	24,000	—
9[e]	22·9	14·6	20×10^6	59·5	38·1	56,000	14·8	9·5	33,000	2·6
	0–41%			41–49%			49–65%			
12[e]	8·9	5·4	—	73·0	44·1	76,000	16·1	9·8	25,000	2·0
13[e]	7·5	4·8	—	69·1	43·6	73,000	23·2	14·7	—	—
14[e]	10·0	6·3	—	73·2	46·3	62,000	10·4	—	—	—

[a] Yield based on 0–65 per cent precipitate No. 3 described in Table II.
[b] Multiply by 0·474 to obtain yield based on weight of sucrose.
[c] 60-gal fermentor.
[d] 300-gal fermentor.
[e] 600-gal fermentor.

the military specifications for clinical dextran. Crude clinical fractions of 41–49 per cent methanol precipitates were preferred for the preparation of purified clinical dextran. At this stage, material of low molecular weight can be obtained from the supernatants by adding additional methanol to 65 per cent concentration; however, this dextran was recovered for characterization purposes only and is not a necessary step in the process. Yields and molecular weights of fractions prepared from native dextran by these procedures are shown in Table III.

Characterization of the molecular-weight distribution of the 41–49 per cent fractions indicated that the lowest 10 per cent of material had a molecular weight of 22,000. Because military specifications require that the lowest 10 per cent of clinical dextran shall have an average molecular weight greater than 25,000, a further fractionation was made. A solution of 41–49 per cent precipitate was diluted with water to 5 per cent dextran concentration, and a 44–65 per cent methanol insoluble fraction was recovered as a semi-fluid gum. This fraction showed a molecular-weight range of 72,000 to 85,000, acceptable for clinical use, and it was obtained in yields of 42–43 per cent of theory, equivalent to a 20 per cent yield based on sucrose used. Yield and characterization of the clinical dextrans obtained in typical runs are shown in Table IV and alcohol precipitation characteristics are depicted graphically in Fig. 5.

An inconsistency occurs between the reported molecular weight for the 41–49 per cent methanol insoluble fraction and the refractionated 44–65 per cent methanol fraction. The 2 per cent loss in yield on refractionation is incompatible with the increase in weight-average molecular weight from 76,000 to 85,000. This inconsistency is attributed to analytical error in determining the yield of the 41–49 per cent fraction. The refractionation probably removes about 10 per cent of the material.

A water solution of the clinical fraction was decolorized with carbon and filtered through clarifying pads in a plate and frame filter. The solution was concentrated in a single effect evaporator to remove residual methanol and spray-dried, using an inlet temperature of 450–460°F and an outlet temperature of 190–200°F; the fine, white powder was collected in an involute Multiclone collector and bag filter.

Table IV. Yield and characterization of clinical dextrans in three typical runs

	Clinical fraction[a]			High fraction[a]			Low fraction[a]		
Yield (%)	43·4[b]	42·5[b]	41·8[b]	9·06[c]	9·24[c]	10·0[c]	8·38[c]	8·39[c]	9·0[c]
M_w	85,000	83,000	72,000	207,000	195,000	113,000	26,000	24,000	26,500
$[\eta]$	0·336	0·334	0·314	0·582	0·570	0·445	0·168	0·166	0·184
$[\alpha]_D^{25}$	201·5	199·7	204	—	—	205	—	—	200
N	0·01	0·01	0	—	—	—	—	—	—
Ash	0	0·03	0	—	—	—	—	—	—
Fructose	0·3	0·28	0·31	—	—	0·18	—	—	0·61

[a] Runs 12, 13, and 14, respectively.
[b] Yield, per cent of theory.
[c] Yield, per cent of clinical fraction.

Dextran with molecular weights meeting military specifications for clinical use was obtained in yields of 43 per cent of theory, equivalent to 20 per cent based on sucrose used (i.e. 20 lb of dextran recovered for each 100 lb of sucrose). Approximately 27 lb of clinical dextran were prepared for clinical testing.

An alternative method of obtaining a clinical dextran, as suggested by Hehre,[4] would eliminate the precipitation of the native dextran. A crude 'central fraction' could be removed from the fermented culture liquid and this portion purified and fractionated. This approach would decrease the quantities of material handled.

During the various stages of fractionation any supernatants not processed further for recovery of dextran were redistilled to recover methanol.

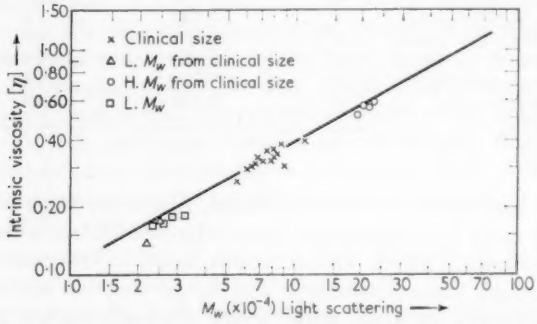

Fig. 6. Only dextrans with a molecular weight between 50,000 and 60,000 will meet present military specification for intrinsic viscosity

Dextran Properties

Intrinsic viscosities and molecular weights for a number of dextran fractions ranging in molecular weight from 22,000 to 228,000 are presented graphically in Fig. 6. From this plot the following relationship was derived:

$$[\eta] = 6{\cdot}51 \times 10^{-4} M_w^{0{\cdot}554}$$

The slope of the curve is greater than that obtained with NRRL B–512 dextrans, and only those with a molecular weight of 50,000

to 60,000 would meet the present military specification of $0{\cdot}23 \pm 0{\cdot}05$, for intrinsic viscosity. It may be noted, however, that a fraction of $M_w = 75{,}000$, the midpoint of the molecular-weight range prescribed by military specifications, has an intrinsic viscosity only 18 per cent above the upper limit for the viscosity specification. The likelihood that this degree of divergence could be of clinical significance is slight.

Periodate oxidation analyses of several fractions of dextran are given in Table V. Since two moles of periodate were consumed

Table V. Characterization by periodate oxidation analysis of dextran fractions

Fraction	Moles IO_4^-/ mole AGU	Moles HCOOH/ mole AGU	Ratio of 1,6-like to 1,3-like units
B-512E (control)[a]	1·93	0·965	27·6
(0–41 per cent ppt.)	1·52	0·741	2·9
(41–49 per cent ppt.)	1·77	0·884	7·6
Clinical	1·77	0·87	6·6
Clinical (high 10%)	1·79	0·849	5·6
Clinical (low 9%)	1·87	0·889	8·0

[a] Clinical.

for each mole of formic acid liberated, each anhydroglucose unit oxidized must have contained three adjacent carbon atoms with free hydroxyls. These would include units in the chain linked only through carbon atoms 1 and 6, as well as non-reducing glucopyranosyl terminal units linked through carbon atom 1. These will be designated '1,6-like' units. The remaining units were not attacked by periodate and therefore must not have had any adjacent carbon atoms with free hydroxyls available for oxidation. Anhydroglucose units linked only through carbon atoms 1 and 3, and giving rise to a linear chain structure, meet this requirement. Several other combinations involving three or more linkages on a unit, and thus producing branched structures, would also not be oxidized. Both types of non-oxidizable units will be designated '1,3-like'.

There appear to be significant differences in the proportion of 1,6-like units in the fractions separated at 0–41 per cent and 41–49 per cent methanol limits. The 0–41 per cent fractions had

approximately 75 per cent of 1,6-like units, whereas the clinical fraction had 87 per cent of this type of unit. The remaining 25 and 13 per cent of the units, respectively, were not oxidized by periodate and were therefore 1,3-like units. Thus, dextran synthesized by NRRL B–1351 is heterogeneous in structure, the higher molecular-weight fractions containing more 1,3-like units.

All fractions have a lower proportion of 1,6-like units than do NRRL B–512 fractions of comparable molecular weight. Van Cleve *et al.*[20] have established from methylation data that the remaining glucopyranosyl units in B–512 dextran, comprising 5 per cent of the total, are linked through carbons 1, 3, and 6 and thus comprise branch points in the molecule. If it is assumed that the 1,3-like units in B–1351 dextran also constitute branch points in the molecule and thus cause a more compact structure, these dextran fractions would be expected to have lower intrinsic viscosities than B–512 fractions of equal molecular weight. Experimentally, the opposite has been found to be true, i.e. the viscosity–molecular weight relationship suggests that B–1351 dextran molecules are actually more extended than those of B–512. It is thus apparent that if 5 per cent or more of the 1,3-like units in the B–1351 molecule comprise branch points, the resulting branched structure must be different to that of the B–512 dextran. The data also permit the possibility that no branching occurs in the B–1351 dextran.

The curves in Fig. 5 indicate the presence of a bi-modal distribution in the native dextran produced. The high mode had a molecular weight in the millions, as shown by the values given in Table III for fractions precipitated at 42 per cent methanol, whereas the low mode had an average molecular weight of less than 100,000. These results are similar to those obtained in a controlled synthesis employing dextran–sucrase from the NRRL B–512 strain.[10]

Fructose content listed in Table VI for a series of fractions of different molecular weight decreases as the molecular weight increases. Hehre[12] has presented evidence to show that molecules of this dextran terminate with a sucrose group. On the assumption that there is one fructose residue per molecule, number-average molecular weights, M_n, were calculated. M_n values are consistent with the breadth of the molecular-weight

Table VI. Fructose content, M_w, and calculated number-average molecular weights, M_n, of dextran fractions

Fraction	No. samples	Average fructose, %	Average M_w	Average M_n (calcd.)	M_w/M_n
0–65% ppt.	2	0·37	—	—	—
0–42% ppt.	6	0·14	42,800,000	135,600	—
41–49% ppt.	4	0·305	77,300	53,400	1·45
42–50% ppt.	8	0·318	79,300	53,900	1·47
Clinical	3	0·30	82,000	54,600	1·50
50–65% ppt.	6	0·67	27,700	25,000	—

distribution expected for the middle fraction. For example, the average M_n value was 53,900 for the 42–50 per cent, 41–49 per cent, and the clinical fractions (Table VI), compared with an average M_w value of 79,300. The average value of the ratio M_w/M_n for these fractions was 1·47, which is somewhat lower than the average value of 1·64 found for clinical fractions of B–512 dextran prepared by acid hydrolysis and fractionation.[6]

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Sewage Sludge Conditioning with Sulphur Oxidizing Bacteria

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Summary. Previous research has shown that a reduction in pH of digested sewage sludge considerably improves its filtration characteristics. Experiments with sulphur oxidizing bacteria (thiobacilli) have shown that under aerobic conditions with a supply of elemental sulphur the pH can be reduced by microbial sulphuric acid synthesis. A semi-continuous process of sludge conditioning by this method was evolved, which would continue if the pH did not rise above pH 7 and if sufficient sulphur was supplied. Attempts to substitute sodium thiosulphate, or sulphide-rich sludge, for elemental sulphur were unsuccessful. A note is appended on a method of enumerating the thiobacilli.

Introduction

The de-watering of sewage sludge derived from anaerobic digestion is often accomplished by filtration through cloth, leading to the separation of a sludge cake and a filtrate. The ease with which this filtration can be accomplished largely controls the economy of the process and the quality (water content) of the cake.

Coackley (1956) proposed the adoption of the specific resistance concept for expressing the ease with which sludges could be filtered, and he developed an experimental technique for its determination. He showed that acidification of the sludge with a mineral acid greatly lowered the specific resistance thus improving its filtration characteristics. His experiments led to the conclusion that the effect was due to acid conditions altering the nature of the sludge proteins, which were most responsible for difficult filtration.

As an alternative to lowering the pH by the addition of acid, experiments were undertaken to determine if biological production of acid would have the same effects. To this end the growth of sulphur oxidizing bacteria was employed.

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Sulphur Oxidizing Bacteria

Three species of autotrophic aerobic sulphur bacilli were obtained from the National Collection of Industrial Bacteria, maintained by the Department of Scientific and Industrial Research. These were *Thiobacillus thio-oxidans*, *Th. concretivorus* and *Th. thioparus*, of which the first two were principally utilized.

These thiobacilli are Gram-negative straight rods, requiring aerobic conditions in an inorganic medium containing sulphur or sulphur compounds. The basal medium for maintaining the growth of *Th. thio-oxidans* and *Th. concretivorus* was composed of (per litre):

$(NH_4)_2SO_4$	0·2 g
$MgSO_4$	0·5 g
KH_2PO_4	3·0 g
$CaCl_2$	0·25 g
Elemental S as a layer on liquid surface	0·25–2%

This medium had a pH of 4·5 and was sterilized by steaming on three successive days.

A method for counting the number of viable sulphur oxidizing bacteria in the experiments is given in the Appendix.

Digested Sewage Sludge

The sludge resulting from anaerobic digestion, used for the experimental work, was obtained from the Mogden Treatment Works of the West Middlesex Main Drainage Department.

For certain experiments, high sulphide content digested sewage sludge was obtained from the Microbiology Group of the Department of Scientific and Industrial Research at Teddington. A description of the production of this sludge has been given by Butlin *et al.* (1956).

Certain characteristics of the sludges are given in Table I. When necessary sludge was rendered sterile by autoclaving at 10 lb/in^2 for 30 min on two successive days.

Preliminary Experiments

Initial experiments were made to determine if the thiobacilli growing in the basal medium would reduce the pH by acid production in the presence of sterile or non-sterile sludge.

Table I. Average characteristics of digested sewage sludges

	Mogden digested	High sulphide digested
pH	7·3	7·0
Specific resistance, c.g.s. units	$1·8 \times 10^{10}$	$1·2 \times 10^{10}$
Solids/100 ml wet sludge	4·0 g	5·0 g
Soluble sulphide/100 ml sludge	5 mg	56 mg
Insoluble sulphide/100 ml sludge	15 mg	17·5 mg

It can be seen from Fig. 1 that the pH was reduced by the growth of *Th. concretivorus* to pH 2 in 20 days in the presence of sludge. The addition of sludge raised the initial pH and consequently the pH reduction lagged behind that for pure basal medium. Similar results were obtained with *Th. thio-oxidans*.

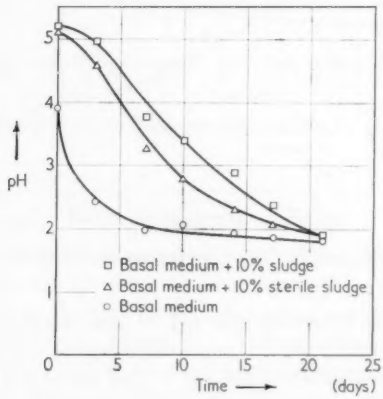

Fig. 1. pH during growth of *Th. Concretivoru* at 30°C

The cultures were incubated as shallow solutions allowing a large surface for diffusion aeration.

Further experiments with deep cultures of basal medium aerated by rising air bubbles from a diffuser showed that active growth of the cultures took place as indicated by bacterial counts and pH measurements. It was noted that *Th. concretivorus* produced

a more rapid pH fall than *Th. thio-oxidans*, and that the former caused sulphur to appear as a suspension in the medium, whereas the latter gave rise to sulphur deposits on the aeration vessel wall. Experiments also indicated that the pH reduction took place more rapidly at 30°C than at 18°C. Later experiments could not generally take advantage of this fact due to the limited incubation accommodation in the laboratory.

It was then thought advisable to grow the thiobacilli as a mixed culture in aerated sludge with the addition of free sulphur but no other elements of the basal medium. Fig. 2 shows the

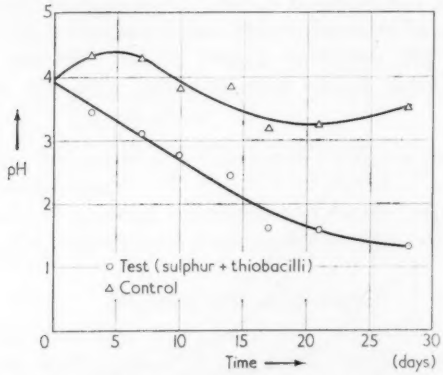

Fig. 2. pH in sludges during aeration at 18°C

result of this test in which the pH of the thiobacilli-inoculated sludge has dropped to pH 1·3 in 28 days compared with a drop to pH 3·5 in the uninoculated control. The initial pH of the sludge was 5·8 which was reduced to pH 4·0 by the addition of sulphuric acid to both test and control sludges. After 11 days the specific resistance of the test sludge had dropped to a third of that of the control. Centrifuging of the two sludges after 17 days produced a clear centrifugate from the test sludge but a cloudy centrifugate from the control. This experiment was conducted with digested sludge which had been aerated for several weeks beforehand.

Because of the anaerobic nature of normal digested sludge, a similar experiment was conducted with freshly digested sludge

straight from the sewage works. It was found necessary to make two additions of sulphuric acid and a second inoculum of thiobacilli before the culture established growth in the test tank. After 10 days pH fell rapidly in the test tank reaching pH 2 after 40 days and pH 1·5 after 60 days. Meanwhile the uninoculated control tank pH had risen first to over 6 and later fell slowly to pH 3 after 80 days. These results are shown in Fig. 3.

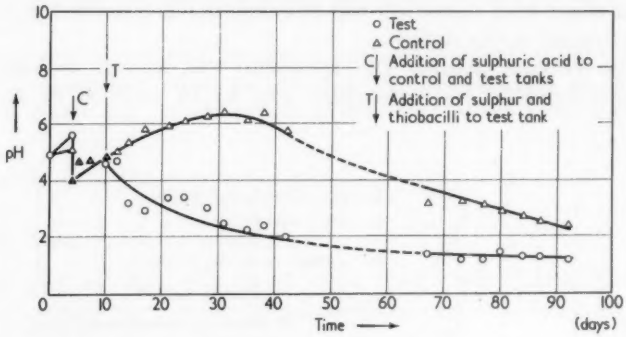

Fig. 3. pH in sludges during aeration at 18°C

Batch Treatment of Sludge

Having shown that active growth of thiobacilli was possible in digested sewage sludge with an accompanying reduction in pH, it was necessary to show that there was an accompanying reduction in specific resistance to filtration. This was verified with sludge to which had been added 40 g sulphur/100 g sludge solids. This sludge was first treated with sulphuric acid to reduce the pH to approximately 4, so that the thiobacilli could establish growth. Both the sludge under test and a control were continuously aerated at 18°C, and the control was acidified to keep its pH in step with the test batch. Weekly determinations of specific resistance were made; the results (shown in Fig. 4) indicated that the specific resistance of the sludge containing thiobacilli was generally slightly lower than that of the control sludge at the same pH. No immediate explanation of this was found, but it

was thought that the metabolism of the dense population of thiobacilli created effects other than the production of acid. The sludge proteins, causing difficult filtration, can be altered not only by pH changes, but to a lesser extent by changes in other ions. Such changes may have been produced by the thiobacilli.

The figure of 40 g sulphur added per 100 g sludge solids was derived from an experiment which was conducted with varying quantities of added sulphur. This experiment also indicated that inoculation with thiobacilli was not essential as a natural population existed in the sludge.

The sulphur bacillus *Th. thioparus* was known to oxidize thiosulphates in its metabolism and to be capable of active

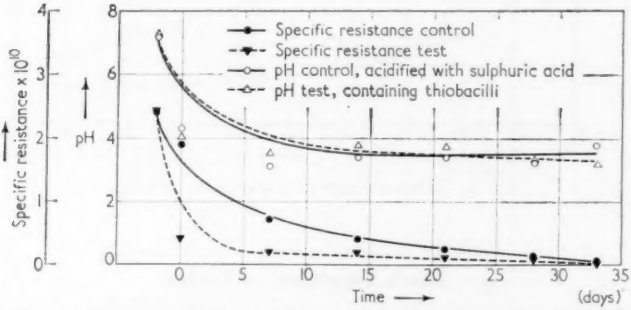

Fig. 4. Specific resistance and pH of sludge during aeration at 18°C

growth at pH 6·5. However, in experiments with sludge acidified to pH 6·5, containing 80 g of commercial sodium thiosulphate per 100 g sludge solids, inoculation with *Th. thioparus* followed by aeration produced no significant growth of the thiobacilli, nor reduction in pH. It was thought that there was probably some reaction between the thiosulphate and the sludge compounds rendering the former unusable by the thiobacilli. However, there has recently been some doubt expressed on the identification of the species supplied by the National Collection of Industrial Bacteria (Townshend, 1960). It may possibly be *Th. thiocyanoxidans*.

Semi-continuous Treatment of Sludge

The results of the batch treatment experiments indicated that the pH reduction rate became appreciably slower when the pH fell below 3. It seemed feasible, therefore, to add further sludge at this point to raise the pH to about 4·5 and so again promote the thiobacilli growth and rapid pH reduction. Withdrawal of some of the low pH sludge, with simultaneous addition of fresh sludge each time the pH fell to 3 would give rise to a semi-continuous process.

This was confirmed with sludge containing added sulphur, the pH of which had fallen to 3·2 after 33 days aeration at 18°C. Fresh sludge but no additional sulphur was added every 6 or 7

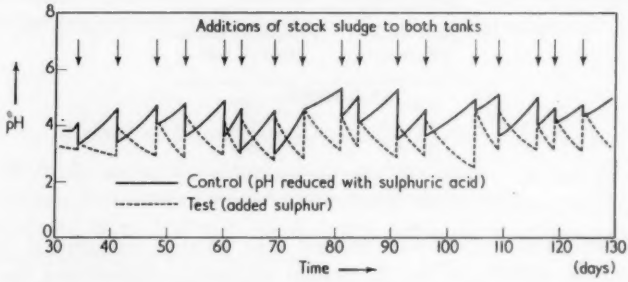

Fig. 5. Intermittent addition of sludge to thiobacilli-active sludge aerated at 18°C

days to raise the pH to about 4·5. A control experiment (i.e. one containing no sulphur) was maintained in which sludge was added at the same time as in the test sludge. Sulphuric acid was added to the control to keep its pH in step with that of the test sludge. The experiment was entirely satisfactory as shown by the results in Fig. 5, where it can be seen that the process was maintained for 100 days after the first addition of fresh sludge. Generally, the specific resistance of the withdrawn samples was about one twentieth of that of the fresh sludge, although this depended to some extent on the nature of the fresh sludge.

A further experiment conducted with aeration at 30°C showed that this higher temperature led to a more rapid rate of pH

reduction. This allowed more fresh sludge to be added, and in a 14-day test, the 30°C sludge treated nearly 2·5 times as much fresh sludge as the 18°C sludge.

The experiments on the semi-continuous treatment process showed that the amount of fresh sludge that could be treated was limited by the fact that too much added sludge would raise the pH to a value where the thiobacilli would no longer actively grow. This had been set at pH 4·5, but a further set of experiments showed that the pH could be allowed to rise to pH 7 with advantage in the quantity of fresh sludge treated. This series conducted at 30°C with aeration gave the results that for a 13-day period the following amounts of fresh sludge were treated if the pH was raised to the range indicated:

Upper limit of pH range	Volume of sludge added, ml[a]
4·0–4·5	480
5·0–5·5	390
6·0–6·5	860

[a] The original volume of sludge in each case was 500 ml.

If the semi-continuous process was carried on over a long period the activity of the thiobacilli ultimately diminished due to shortage of sulphur. No critical evaluation of the quantities required was made although measurements of the titrable acidity (as H_2SO_4) would enable the quantities of sulphur used to be derived stoichiometrically.

Utilization of High Sulphide Sludge

Certain experiments on sewage conducted by the Microbiology Group of the D.S.I.R. at Teddington resulted in the production of a sludge rich in sulphide content. Experiments conducted to establish the growth of thiobacilli in this sludge, without the addition of elemental sulphur, were unsuccessful. The sludge was aerated at 30°C in the normal way. However, when elemental sulphur was added there was a gradual fall in pH indicating the active growth of thiobacilli.

Conclusions

The experiments concerning the growth of sulphur oxidizing bacteria in digested sewage sludge indicated:

(1) The specific resistance to filtration of the sludge falls as the pH is diminished, independently of whether the pH reduction is obtained by acid addition or by microbial activity.

(2) A low pH in the sludge can be produced by aeration with the addition of an optimum of 40 g of elemental sulphur per 100 g of sludge solids.

(3) This process proceeds more rapidly at 30°C than at 18°C.

(4) The process can be made semi-continuous by the addition of increments of fresh sludge to raise the pH to a limit of pH 7·0.

(5) Further additions of sulphur are required as the supply becomes exhausted. Sodium thiosulphate and sulphide-rich sludge are not effective substitutes for elemental sulphur.

Appendix

Method of Enumerating Sulphur Oxidizing Bacteria

Multiple serial $\frac{1}{10}$ dilutions of the liquid to be tested were inoculated into an inorganic medium and the most probable number evaluated from McCrady probability tables as used for coliform enumeration in water samples.

Growth of the sulphur oxidizing bacteria in the medium was detected by the colour change, due to acid production, of methyl orange indicator in the medium.

Medium used:

NH_4Cl: 0·1 g
K_2HPO_4: 0·25 g
KH_2PO_4: 3·0 g
$MgCl_2$: 0·1 g
$CaCl_2$: 0·1 g
Sulphur: approximately 0·1 g per 10 ml of medium
Distilled H_2O: 1,000 ml
Methyl orange (0·04 per cent solution): 5·0 ml
pH = 5·6; sterilization by steaming on three successive days for 30 min; incubation for 10 days at 30°C

Acknowledgment. The work described in this paper was carried out by the senior author (Wendye E. Johnston) in the Chadwick Public Health Engineering Laboratory at University College, London, under the direction

of Mr. H. R. Oakley and Professor H. J. Collins. The material has been abstracted and prepared for this paper by the junior author (K. J. Ives) from Research Reports Nos. 3A and 7 of the Department of Civil and Municipal Engineering, University College, London.

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Analytical Control of the Biological Oxidation in the Production of $\Delta^{1,4}$-Androstadiene-3,17-dione

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Summary. A rapid method for the assay of $\Delta^{1,4}$-androstadiene-3,17-dione has been worked out for use in the control of its production by biological oxidation. The method consists of a single selective extraction step, a specific colour reaction and a photometric measurement. Based upon the analyses of samples taken at frequent intervals, the oxidative process can be stopped at its optimum production level. Thus insufficient transformation and over-oxidation can both be avoided. The time needed for one analysis is 20 minutes.

Introduction

Instead of isolating it from horse urine,[1, 2] a new method has recently been found for the production of oestrone. This method is based on Inhoffen's researches[3, 4] concerning aromatization, notably of $\Delta^{1,4}$-androstadiene-3,17-dione. This compound can be transformed directly into oestrone. The $\Delta^{1,4}$-androstadiene-3,17-dione can be produced from progesterone or from Δ^{4}-androstene-3,17-dione with the help of micro-organisms.[5–12] The transformation of the $\Delta^{1,4}$-androstadiene-3,17-dione, however, is not uniform and homogeneous: some intermediates and over-oxidized substances are also produced, as was shown by Danish[13] and Japanese[14] authors. The transformation of progesterone, when carried out with *Fusarium caucasicum*, yields the intermediate Δ^{4}-androstene-3,17-dione, besides $\Delta^{1,4}$-androstadiene-3,17-dione, and, as a result of over-oxidation, 1-dehydrotestololactone is also produced. There is maximum concentration of $\Delta^{1,4}$-androstadiene-3,17-dione attainable in the course of this transformation, as shown also by the experiments of Wix and Albrecht.[15]

For the estimation of the steroids produced during these transformations, paper chromatography has been extensively applied;

the methods of Burton, Zaffaroni and Keutmann,[16] and that of Bush[17] may be mentioned as the fundamental ones. For the rapid quantitative determination of these compounds, Albrecht and Wix[18] recently described a method. However, even by this method the time required for one analysis amounts to several hours. Thus with the help of chromatography, information about the course of the transformation can be had only at the close of the fermentation process.

The method presented in this paper for the quantitative control of the transformation process by the analysis of samples taken at short intervals is based upon selective extraction, a specific colour reaction and photometric measurement.

Experimental

The fermentation sample contains in varying proportions the four steroids mentioned in the introduction and several impurities of the fermentation broth; thus the disturbing effect of the latter must also be taken into account. Examinations of the effect of these impurities have shown them to be of a polar character, and it has been found that their disturbing influence can the better be counteracted the less polar the solvents which are chosen for extraction; carbon tetrachloride proved to be the most suitable. Carbon tetrachloride does not dissolve the substances which disturb the analysis of the fermentation samples. When four parts by volume of this solvent are used for one volume of the fermentation sample a practically complete extraction of $\Delta^{1,4}$-androstadiene-3,17-dione can be achieved in a single step. Without any purification of the organic phase, evaporation of an aliquot to dryness yields the steroids in the form of a colourless residue.

Following this, the possibilities of the estimation of the $\Delta^{1,4}$-androstadiene-3,17-dione content of this steroid mixture have been investigated. Ultraviolet absorption in ethanol of these four steroids is characterized by curves with nearly identical maxima and general form, as shown in Fig. 1.

Attempts were then made to base the method upon the absorbancies of the chromogens formed with concentrated

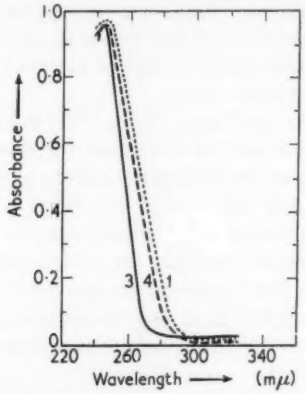

Fig. 1. Ultraviolet absorption spectra of progesterone (1), $\Delta^{1,4}$ androstadiene-3,17-dione (3), and 1-dehydrotestololactone (4) in absolute ethanol; in 10-mm cuvettes, concentration 10 μg/ml

sulphuric acid, since these values showed somewhat more pronounced differences. However, the curves appertaining to these derivatives are still too superimposed upon each other to allow them to be used for purposes of analysis (Fig. 2).

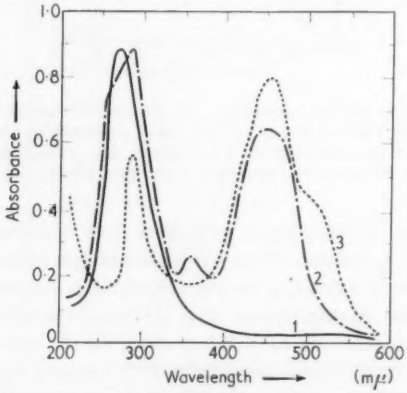

Fig. 2. Absorption spectra of the chromogens, formed with concentrated sulphuric acid, of progesterone (1), Δ^4-androstene-3,17-dione (2), and $\Delta^{1,4}$-androstadiene-3,17-dione (3); in 10-mm cuvettes, concentration 200 μg of the steroids in 10 ml of concentrated sulphuric acid solution

With proper variation of the concentration of sulphuric acid, temperature and duration of the reaction, and with the addition of different substances (salts of iron or mercury, etc.) it is possible to obtain more divergent curves.

The reaction of Kober[19] and Haenni[20] is used for the estimation of oestrone. Carried out as described by Banes,[21] this reaction furnishes chromogens the absorption curves of which show marked differences from each other, e.g. for the chromogen characterizing the $\Delta^{1,4}$-androstadiene-3,17-dione, λ_{max} = 4,650 Å. In order to shorten the time of reaction for which originally[21] 2 h were stipulated at 70°C, this temperature has been raised. The extinction values at λ = 4,650 Å of the chromogen produced at 100°C are plotted in Fig. 3 as a function of reaction time. This shows

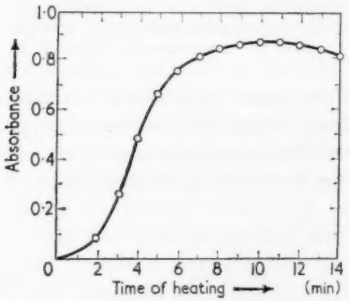

Fig. 3. Absorbency of the chromogen of $\Delta^{1,4}$-androstadiene-3,17-dione as a function of heating time at 100°C. Chromogen prepared by the Kober–Haenni reaction. Extinctions measured at λ = 4,650 Å, in a 10-mm cuvette. Concentration 200 μg of the steroid in a reaction mixture of 11 ml

that maximum development of colour was obtained in 10 min. The steroids in question, when subjected to the same treatment in the same concentrations, give the absorption curves presented in Fig. 4. At the maximum of the $\Delta^{1,4}$-androstadiene-3,17-dione curve the other steroids do not interfere, or if they do, this effect is negligibly small. This is especially true around optimum values of production when, compared with the concentration of the chief product, that of the other steroids is distinctly inferior.

The extinction measured at λ = 4,650 Å, nearly attaining the

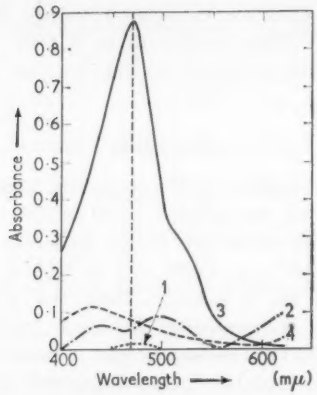

Fig. 4. Absorption spectra of the chromogens (by Kober–Haenni reaction) of progesterone (1), Δ^4-androstene-3,17-dione (2), $\Delta^{1,4}$-androstadiene-3,17-dione (3), and 1-dehydrotestololactone (4). Reaction time = 10 min. Extinctions measured at λ = 4,650 Å in a 10-mm cuvette. Concentration of the steroid = 200 μg in 11 ml of the reaction mixture

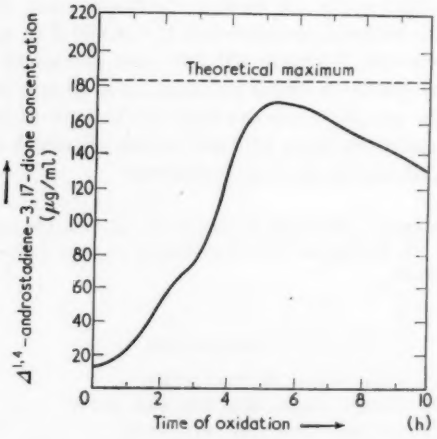

Fig. 5. Changes of the concentration of $\Delta^{1,4}$-androstadiene-3,17-dione during oxidative fermentation

theoretical value, shows a maximum within the duration of a fermentation run. This maximum is a true indication of the best production level, as shown in Fig. 5.

Procedure

From the fermentation broth originally containing 200 μg of progesterone per ml, an aliquot (2·00 ml) is transferred into a glass-stoppered test tube of 15-ml capacity and 15-mm o.d. Carbon tetrachloride p.a. (8·00 ml) is added, the test tube is vigorously shaken for a minute, then the phases are allowed to separate. The fermentation liquid is removed by suction and from the carbon tetrachloride phase a part (4·00 ml) is transferred into another glass-stoppered test tube of 15-ml capacity, where it is evaporated to dryness by immersion in a boiling-water bath. The last remnants of the carbon tetrachloride are expelled by a gentle stream of nitrogen introduced into the test tube. The dry residue is taken up in absolute ethanol (1·0 ml), and to this solution diluted Kober–Haenni reagent (10·0 ml, prepared by Banes's method[21]) is added. After homogenization, the test tube, its stopper sitting loosely, is kept in boiling water for 10 min. After cooling, the extinction of the yellow solution is measured at $\lambda = 4{,}650$ Å in an appropriate spectrophotometer (Unicam SP 500 and Beckman D.U. in our experiments) against a blank solution, in a 10-mm cuvette. The concentration of $\Delta^{1,4}$-androstadiene-3,17-dione is taken from a calibration curve showing plotted values obtained with different ethanolic solutions of the pure substance.

Acknowledgments. We wish to thank Dr. G. Wix for valuable suggestions, and Dr. M. Rados for the preparation in pure form of the steroids used in this work.

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Purification Process Development using Radioactive Vitamin B_{12}*

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Summary. The use of low-level radiotracer techniques permits rapid, precise estimation of yields and material balances for processing steps. This is a great advantage for development work with Vitamin B_{12} because the conventional methods using microbiological assays are slow and lack precision. Release of the vitamin from *S. griseus* cells, selection of absorbing resins, and screening of eluting agents were followed using cobalt-60 cyanocobalamin. The method is most valuable when supplemented by microbiological assays because the yields of radioactivity are misleading in conditions which inactivate the vitamin.

Introduction

The conventional methods for process development in the purification of Vitamin B_{12} from streptomycete fermentations use microbiological response assays with *Lactobacillus leichmanii*. Depending upon how well the dilutions have been estimated, results are obtained from 30 hours to seven days after submission of the samples. The usual lag before experiments can be evaluated is about four days. Furthermore, the microbiological assay lacks precision; 5 per cent precision is rarely obtained, and about 10 to 15 per cent is normal. When searching for small yield improvements, this time lag and lack of precision cause purification development research to be slow and tedious. A precise, accurate and fairly rapid radio-dilution assay may be used, but it is far too expensive for routine work.

The structure of Vitamin B_{12} is shown in Fig. 1. The molecule is roughly spherical with the cobalt atom at the centre. 'X' denotes various anions such as hydroxide, cyanide, and chloride which can be complexed to the cobalt atoms to give different

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vitamin factors. These can be resolved by paper chromatography and show marked differences in chemical stability. Other known forms of the vitamin arise from modifications of the benzimidazole grouping. Adenine at this position gives pseudo-Vitamin B_{12}, which is active in some micro-organisms, but not in higher animals. A number of patents have appeared claiming the addition of substituted benzimidazoles to produce new vitamin derivatives biosynthetically. None of these seem to have commercial importance as yet.

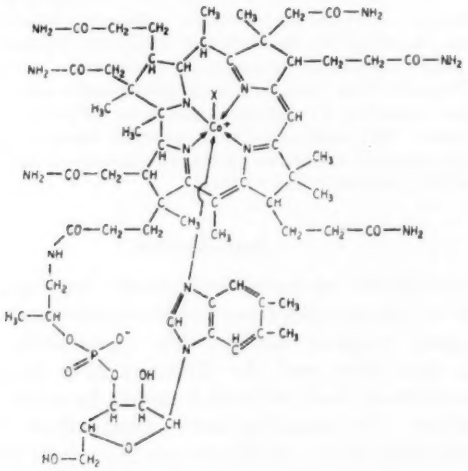

Fig. 1. Vitamin B_{12}

The nomenclature of compounds of this type has been discussed recently,[1] and cobamide is the name designated to the parent compound. For convenience, the older and more customary terms such as cyanocobalamin instead of 5,6 dimethyl α-benzimidazoyl cobamide cyanide will be used here.

Radioactive Vitamin B_{12} in the form of cobalt-60 cyanocobalamin has been prepared by adding labelled cobalt compounds to fermentations.[2,3] Pure crystalline cobalt-60 cyanocobalamin is available commercially.[4] While there has been a great deal of

tracer experimentation on the absorption and metabolism of Vitamin B_{12}, little published work has appeared using tracers for research on its purification. Its radioactivity has been used to locate the tagged vitamin in paper electrophoresis separations[5,6] and in a counter-current distribution series,[7] but no quantitative yield comparisons based on radioactivity were found in the literature.

Three basic problems in possible preliminary purification steps for Vitamin B_{12} were studied by tracer techniques in our laboratory. These were: (*a*) release of the bound vitamin from the cells in the fermentation broth; (*b*) selection of a resin to absorb the vitamin; and (*c*) screening of various elution conditions. Some steps covered by patents were included for comparative purposes.

Experimental

(1) *Broth equilibration*

The factors to be considered in attempting to equilibrate labelled Vitamin B_{12} with the native vitamin in a fermentation broth are: (*a*) about 90 per cent of the vitamin is bound by the cells; (*b*) several forms of the vitamin are present; and (*c*) conditions which favour equilibration would make the system atypical.

Since cell binding presented a very complicated exchange and equilibration problem, the assumption was made that the broth would be satisfactory for use when no further change was noted in the distribution of isotope between the solid and liquid phases. Equilibration was tested by withdrawing samples, centrifuging them and counting the radioactivity in the clear liquid. A scintillation crystal detector in a well counter was used for the measurements. Because of the half-life of about 5 years, the time correction for the samples was negligible. For 200 ml of whole broth, only 0·2 μc of cobalt-60 cyanocobalamin was required to get about 1 per cent counting accuracy, because the gamma radiation of cobalt-60 is so easily measured. Equilibration is shown in Fig. 2. At neutral pH, 24 h seemed to give fair equilibration with about 50 per cent of the activity bound by the solids. The curve for pH 2 showed a faster reaction with 15 per cent binding.

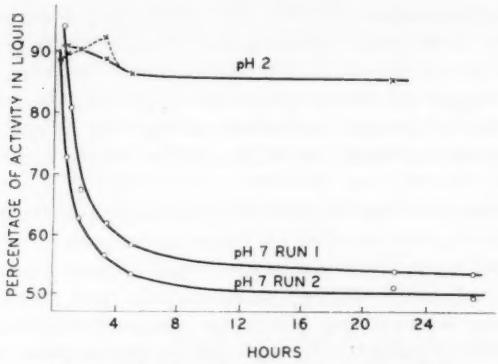

Fig. 2. Equilibration curves for whole broth

(2) *Release from whole broth*

'Equilibrated' broth at neutral pH was acidified in stages and samples were taken for estimation of vitamin release. The results are shown in Fig. 3. Not as much release was noted at pH 2 as would have been predicted from the previous figure showing equilibration at pH 2. Part of this discrepancy may be due to a rate phenomenon, but the conversion to other forms with

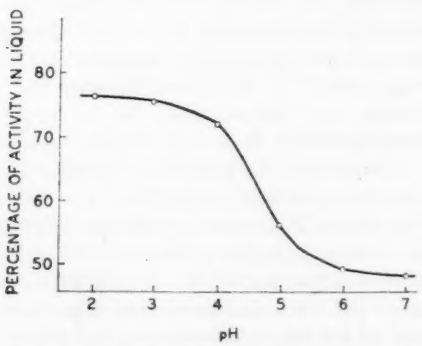

Fig. 3. Release of radioactivity in whole broth

different binding properties could be the principal explanation. Over a 2-h holding period, a small increase was noted in liquid-phase radioactivity, but most of the release occurred in the first few minutes. The effect of temperature was studied by selecting four reasonable release conditions and sampling at time intervals. The amount of radioactivity in the centrifuged supernatant was used to calculate the graphs shown in Fig. 4. The most severe conditions, pH 2 at 80°C, released over 90 per cent of the radioactivity. The other conditions gave lesser amounts. The decline in the released vitamin with longer holding may result from pre-

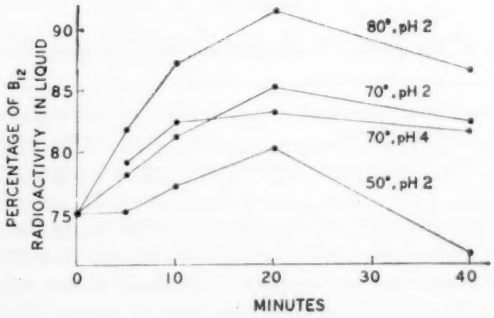

Fig. 4. Release of Vitamin B_{12} from whole broth

cipitation of degraded forms or co-precipitation on denatured proteins. Comparison of these data with microbiological assay data permits study of both release and stability.

(3) *Adsorption on resins*

Modern techniques for adsorption on resins use counter-current operations with columns or with successive batch contacts. For fundamental investigations of the mechanisms involved, radioisotopes offer the advantages of rapid quantitative assay and the possibility of measuring the test compound while still bound to the

resin. The present problem, however, was to select a resin from the large number available, and batch-wise testing seemed far less complicated than running a large number of columns. Batch-wise tests were planned using the following criteria: (*a*) rate of adsorption; (*b*) total adsorption; and (*c*) over-all yield including elution. Ten-millilitre samples of a hydrogen- or chloride-form resin were stirred with 300 ml of broth processed to this point by conventional methods and then equilibrated with added labelled vitamin. Samples of the liquid phase were withdrawn after certain time intervals and counted for radioactivity. A check was obtained with one resin on the amount of absorbed radioactivity calculated from solution differences by counting directly a sample of resin in the well counter. The good agreement in Table I

Table I. Comparison of measured and estimated resin activity

Calculated from solution difference:	$5{\cdot}30 \times 10^4$ counts/min/5 ml
Direct counting of resin:	$4{\cdot}82 \times 10^4$ counts/min/5 ml

shows that attentuation of the gamma radiation by the resin was not severe. The resins which adsorbed the vitamin were eluted with aqueous sodium bicarbonate. Table II shows some of the results. Carboxylic resins were the best of those tested. Some typical rate curves are shown in Fig. 5. Only small differences in adsorption rate and elution yield were noted and thus were discounted in selecting the resin.

Table II. Comparison of resins

Resin	Type	Percentage of radioactivity absorbed	Elution yield
IRC–50	Carboxylic	87	91
XE–89	Carboxylic	78	96
IR–120	Sulphonic	9	—
IRA–410	Quaternary amine	0	—
Duolite–A–114	Amine	2	—

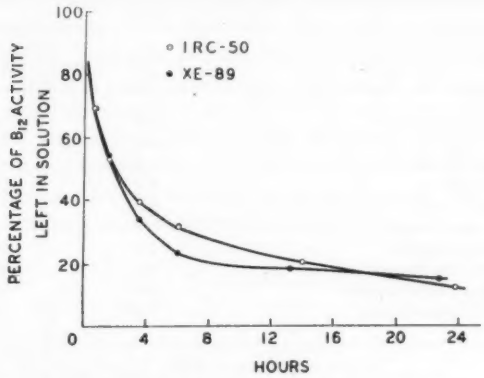

Fig. 5. Adsorption of Vitamin B_{12} on various resins

(4) *Screening of eluants*

Tracer techniques were particularly useful in screening of eluants to strip the adsorbed vitamin from the resin. It is an advantage to measure radioactivity instead of microbiological activity in this work because elution of the molecule is distinguished from its stability. Unless degraded forms interfere with or compete with the desorption, it is possible to study directly the removal of activity from the resin. The most promising agents can then be studied in the presence of such stabilizers as cyanide ion with microbiological assays as the criteria for the optimum system.

A large batch of loaded resin was prepared by adding 175 ml of fresh resin to 9 l. of filtered broth which had been equilibrated for 20 h with 2 μc of cobalt-60 cyanocobalamin. Absorption as a function of time will be shown later.

Ten-millilitre samples of loaded resin were eluted with 100-ml portions of different eluants. The data are shown in Table III. The bicarbonate and phosphate ions were found to elute the radioactivity, and pH was not the critical factor because sodium hydroxide was a poor eluant. Under different circumstances, sodium hydroxide was much better; this will be discussed later.

A serious problem at this juncture was to be sure that the labelled vitamin was a true indicator for piloting a plant operation.

Table III. Batchwise elution of tagged cyanocobalamin from IRC–50 resin

Eluant	pH	Percentage of radioactivity eluted
10% Trisodium phosphate	8·2	93
Sodium hydroxide to	5·92	19
Sodium hydroxide to	7·10	37
Sodium hydroxide to	8·05	30
1%sodium bicarbonate	6·5	74
2·5% sodium bicarbonate	6·8	95
5% sodium bicarbonate	7·2	99
1% ammonium bicarbonate	6·4	75
2·5% ammonium bicarbonate	6·8	97
5% ammonium bicarbonate	7·25	99

Counter-current column techniques would best approximate plant conditions, but this complicates radiochemical operations by contaminating hoses, funnels, screens and other equipment. The chance of spills is also much greater because of flow control from reservoirs to columns and to waste receivers. It was not felt that the extra trouble and risks were justified for these screening experiments. The alternative was to attempt to equilibrate labelled vitamin with the native vitamin already on resin loaded by column techniques.

Resin from the same source as the fresh resin of the previous experiment, but loaded by counter-current column operations, was stirred with an aqueous solution of cobalt-60 cyanocobalamin. This allowed equilibration with Vitamin B_{12} already on the resin. The absorption rate is compared with that for fresh resin in Fig. 6. The saturated resin absorbed radioactivity much faster, suggesting that a different mechanism is operating. One explanation that has been proposed but not tested is that adsorption on fresh sites is a slower process than the displacement of a molecule already adsorbed on the resin. Another important difference would be the effect of other adsorbed molecules; the vitamin may be bound to some macromolecule instead of finding a site directly on the resin. Though there was uncertainty concerning the equivalence of the labelled and native vitamin, it was decided to proceed with the screening experiments. The assumption was that promising

eluants from these tests would warrant extensive study using column techniques.

The samples from this second batch of resin were subjected to multiple elutions, each including one hour of contact time. Elutions were terminated by decanting the solution from the resin. The physical separation was not sharp because duplicate runs gave about the same total yield, but had different proportions in each elution. Of the nineteen runs performed, only those with pertinent information are listed in Table IV.

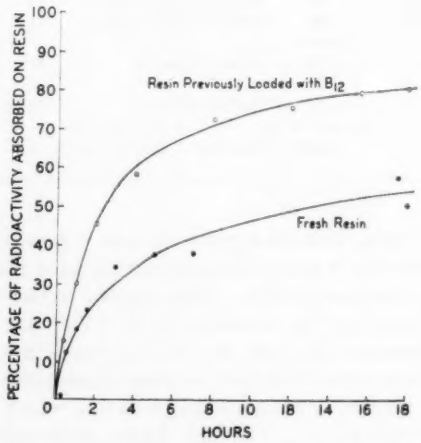

Fig. 6. Comparison of fresh resin and loaded resin

The yields in excess of 100 per cent were caused by errors in measuring only 10 ml of wet resin in graduate cylinders. Shape factors affect packing in small containers, and the investigator tends to estimate his resin volume on the low side because he lacks confidence in the resin packing. This could have been eliminated by practising with the small graduate and verifying multiples in a larger graduate.

Note that the bicarbonate compounds continued to show promise as eluting agents. An important difference, however, was noted with sodium hydroxide. In the runs with labelled vitamin

Table IV. Batchwise elutions of radioactivity

1st Elution Agent	Yield, %	2nd Elution Agent	Yield, %	Total yield, %
1. $NaHCO_3$	67	$NaHCO_3$	31	98
2. $NaHCO_3$	95	$NaHCO_3$	19	114
3. NH_4HCO_3	93	NH_4HCO_3	14	107
4. NH_4HCO_3	79	NH_4HCO_3	16	95
5. NH_4HCO_3	82	NH_4HCO_3	13	95
6. NH_4OH (pH 7·5)	87	NH_4OH (pH 7·5)	16	103
7. NH_4OH (pH 7·5)	86	NH_4OH (pH 7·5)	11	97
8. Acidic Ethanol	15	Acidic Ethanol	11	26
9. NaOH (pH 8)	76	NaOH (pH 8)	20	96
10. $NaNO_2$	5	Na_2SO_3	1	6

loaded on fresh resin, sodium hydroxide was a poor eluant, but when the radioactivity was equilibrated with loaded resin, sodium hydroxide gave excellent yields. This may be a function of the type of sites occupied by the labelling agent. The rate differences and elution differences of fresh and loaded resins are intriguing problems on which more work will be done when time permits.

Run 10 shows that nitrite ion and sulphite ion, which are known stabilizers of Vitamin B_{12}, do not have appreciable eluting properties.

(5) *Comparison of microbiological yields and radioisotope yields*

In a number of runs, both radioactivity counts and microbiological assays were run on the same eluate fractions. It was permissible for the regular assay personnel to handle the samples because preliminary dilutions to assay levels in the radiochemistry laboratory reduced the radioactivity to negligible amounts. The results are given in Table V. The important point is that the tracer yield may be higher, the same as, or lower than the microbiological yield.

Table V. Comparison of batch elution methods

	Eluant	Tracer yield	Bio-yield
Runs 1 and 2			
1st Elution	10% $NaHCO_3$	63 and 90	53 and 87
2nd Elution	10% $NaHCO_3$	20 and 7	29 and 7
3rd Elution	10% $NaHCO_3$	1 and 0	10 and 0
Total		84 and 97	92 and 94
Run 3			
1st Elution	$NaHCO_3$	60	29
2nd Elution	NaOH pH 10	31	27
3rd Elution	NaOH pH 10	8	18
Total		99	74
Runs 4, 5 and 6			
1st Elution	NH_4OH (pH 9·7)	98, 72 and 94	93, 62 and 107
2nd Elution	NH_4OH (pH 9·7)	7, 25 and 5	13, 25 and 0
3rd Elution	—	—	—
Total		105, 97 and 100	106, 87 and 107
Run 7			
1st Elution	60% acidic acetone	19	24
2nd Elution	60% acidic acetone	28	50
3rd Elution	60% acidic acetone	13	13
Total		60	87

Bicarbonate eluants consistently removed the radioactivity in high yields but gave erratic yields of microbiological activity. This was found to result from chemical instability of the vitamin under these conditions.

Ammonium hydroxide gave a consistently good yield by both methods of determination, and the chemical stability was found to be good if the eluates were adjusted to a more favourable pH range.

The most puzzling feature was the failure to get good recoveries of radioactivity with acidic acetone. The respectable yields of microbiological activity agreed with previous experience. Acidic ethanol, shown in Table IV to be poor in eluting radioactivity,

was also known to give fairly good yields of microbiological vitamin activity.

Two explanations were proposed for the differences in yields. The cyanocobalamin probably went on the resin with little equilibration with other forms. Thus, the elutions may have detected physical chemical differences in adsorption. Since only acidic solvents showed this effect and interchange of forms is slow at low pH, an explanation in terms of the properties of the cyano form seems reasonable. The alternative proposal was that the evident difference in mechanism in adsorbing on loaded resin may have been reflected in a difference in elution mechanism. Both of these ideas could have been explored by continuing the experimental programme, but it was decided to stop and evaluate the leads already obtained.

Conclusion

Radiotracer techniques have been shown to have particular value for purification development studies with the labile biochemical Vitamin B_{12}. Leads obtained from tracer experiments must be verified by microbiological assays, but yield discrepancies may provide clues to the process mechanisms. The savings in time and expense, the precise material balances, and the added insights into mechanism are the rewards of using radiotracers in process development.

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Pilot Plant Studies on Parboiling of Rice—I Soaking and Gelatinization

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Summary. The conventional parboiling process used in the Orient consists of steeping paddy in cold or lukewarm water for 48–72 hours, steaming for 15–25 min and then drying in the sun. This process yields rice of a poor quality, and an attempt has been made to improve it by devising both batch and continuous processes in which the soaking and steaming are carried out in the same equipment. The processes developed give a larger throughput and also a product of a quality superior in appearance, flavour and thiamine content to that produced by the usual parboiling method.

Introduction

Rice is the staple food for the vast bulk of the peoples of Asia, whose diet is generally of low nutritive value. Hence every care should be taken to retain the nutritive value of rice during processing. If paddy (rice in husk) is milled without pre-treatment, the 'raw' rice produced loses most of the vitamins present in unmilled rice.[1–3] In the East, however, a considerable quantity of paddy is parboiled (*vide infra*) and dried before milling. Though parboiling had been devised apparently empirically in olden times, it has some very beneficial effects. If properly carried out, it improves the conservation of not only the water-soluble vitamins of the B-group but also of protein and minerals of nutritional importance.[4] The technical advantages of parboiling are greater ease of shelling, better keeping quality, better retention of shape by cooked grains and a 5–10 per cent greater yield of head rice.[5] The last mentioned aspect is particularly important in view of the world shortage of rice. It has been calculated that in India, if all the paddy were parboiled, more than half a million tons of additional rice would be available annually for human consumption.

Parboiling is practised both in rural homes and in the rice milling industry. In the usual commercial parboiling process, paddy is soaked in cold or lukewarm water for 2–3 days in rectangular concrete tanks of 15–20 tons capacity with or without changes of water. The soaked paddy is then removed to smaller mild steel tanks of about half a ton capacity and steamed for a short period (15–25 min). The soaked and steamed paddy is then dried in the sun and milled. The resulting parboiled rice is darkish brown and has a marked off-flavour due to the prolonged soaking in water and also sometimes to stacking of soaked and steamed paddy due to unfavourable weather conditions. The product so obtained is of poor quality with low retention of vitamins.[6]

Parboiling has been practised commercially to a small extent in some Western countries where the old Oriental principle is applied in modern plants. The patented methods like those of Malek, Gariboldi and Farnandes[7] differ from the original process in time, temperature and technique of soaking and drying. In the Malek process the paddy is steamed under pressure. The process of Gariboldi employs autoclaves for soaking, steaming and drying. The rice produced by the above processes, however, has usually a 'white belly' (a white core in the centre of the grain) and does not resemble the parboiled rice of the Orient.

The object of the present investigation was to develop controlled, quick and mechanized methods for soaking, gelatinization and drying of paddy so as to produce good quality parboiled rice, acceptable to consumers in the East.

Material and Methods

Material

The paddy used for this study was 'Patnai variety' grown in West Bengal (India), the original thiamine content of which was 3·5–4·0 μg/g of dry paddy. The average size of the paddy grain was 0·41 inches in length, average thickness in the middle was 0·08 inches, and the bulk density was 34·56 lb/ft^3.

Soaking and Gelatinization

It was observed by preliminary studies in the laboratory (unpublished) that soaking of the paddy to the desired extent

could be completed in 2·25–3·5 h at 65–80°C. It was observed that there was no 'white belly' in the rice grains when the moisture content of paddy after soaking was appproximately 50 per cent on the dry basis. It was further noted that the best results in gelatinizing could be achieved by steaming the soaked paddy for 3–5 min in open steam. Keeping in view the above results, different pieces of soaking and gelatinizing (steaming) equipment were designed and fabricated for pilot-plant studies.

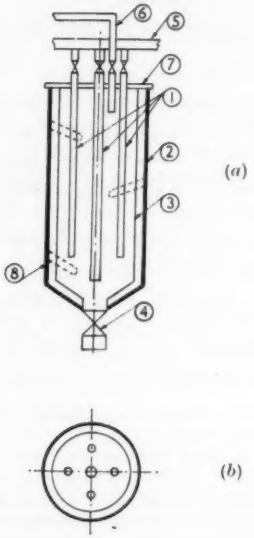

Fig. 1. Batch soaker–steamer for parboiling of rice. (*a*) Sectional elevation: 1, steam pipe; 2, mild steel shell; 3, concrete lining; 4, sluice valve; 5, steam main; 6, water main; 7, top cover; and 8, thermocouple well. (*b*) Sectional plan

Equipment

The soaker–steamers used for this study were of the following two types. One was a batch and the other a continuous one in which both soaking and steaming could be carried out.

(*i*) *Batch soaker–steamer.* It consisted of a vertical cylindrical mild steel tank (Fig. 1, (*a*) and (*b*)), 1 ft in diameter and 5 ft high,

conical at the bottom with a sluice valve for releasing the paddy from the tank and was fitted with five vertical evenly-distributed steam tubes with perforations of $\frac{1}{2}$-in. diameter closed at the bottom. It was fitted with a removable lid at the top, thermometer wells and water and steam connections, and was installed on a platform 3 ft from the ground.

(*ii*) *Continuous soaker–steamer.* The soaker was comprised of a semicircular mild steel trough, 1 ft in diameter and 8 ft long (Fig. 2, (*a*) and (*b*)), jacketted for heating by passing steam or hot

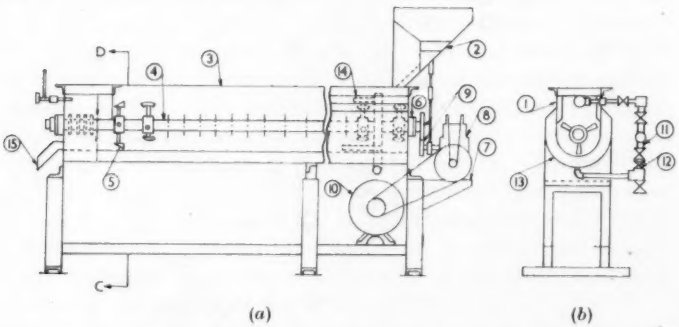

Fig. 2. Continuous soaker–steamer for parboiling of rice. (*a*) Sectional elevation: 1, soaking trough; 2, feed hopper with vibratory plate and adjustable gate; 3, cover for the trough; 4, shaft with mixing and covering paddles; 5, scooper blades; 6, bush bearings; 7, supporting structure; 8, worm gear reducer; 9, spur pinion; 10, electric motor; 11, hot water and steam connection; 12, steam injector water heater; 13, steam jacket for the trough; 14, hot water spray pipe; and 15, discharge spout. (*b*) Section across CD

water through the jacket. At the end of the soaker was a 1-ft long closed trough (steamer), fitted with a screw conveyor, in which gelatinization of rice grains could be carried out by open steam. A horizontal shaft, to which 20 rubber-lined mild steel paddles were attached, was fitted within and along the trough. The inclination of the paddles could be adjusted for meeting the requirements of mixing and conveying. At the end of the soaking zone, 3 scooper-blades were fitted on the shaft to scoop out the soaked paddy into the steaming zone. The soaker–steamer was provided

with an arrangement for hot and cold water spray, a perforated steam pipe in the steaming zone and a drive system for rotating the shaft along with the paddles. The soaker–steamer was inclined in such a way that the water discharged after soaking might not enter into the steaming zone.

The methods for the study of optimum soaking and gelatinization conditions on a pilot-plant scale are given below.

(*i*) *Batch soaker–steamer.* (*a*) The batch soaker–steamer was filled up with approximately 100 lb of water and steam was turned on through steam tubes till the desired temperature was reached. Paddy (approx. 100 lb) was then charged, when the temperature dropped by 5–10°C. The desired soaking temperature was maintained by passing live steam through the tubes as and when required. After soaking for a definite period, the water was drained off by slightly opening the sluice valve and then the rice grains were gelatinized by passing live steam for 3 min. The moisture and thiamine contents of paddy steeped in water at different temperatures and for varying periods were determined. The moisture content was determined by drying the sample in an air oven at 105°C to constant weight and was expressed in percentage on a dry basis. The thiamine content was estimated by the method of Harris and Wang[8] after de-husking and grinding (100 mesh) and was expressed in micrograms per gram of dry rice. The results are given in Table I.

Table I. The moisture and thiamine contents of paddy after soaking and steaming under particular conditions

No. of obs.	Soaking temp., °C	Period of soaking, h	After soaking		After soaking and steaming	
			% Moisture	Thiamine, µg/g	% Moisture	Thiamine, µg/g
1	70	2·5	43·0	2·71	46·5	2·72
2	70	3·0	48·6	2·70	51·2	2·65
3	75	2·5	48·1	2·56	52·1	2·70
4	75	3·0	54·2	2·65	57·6	2·62
5	80	2·5	53·2	2·62	57·0	2·64
6	80	2·5	53·2	2·62	57·0	2·64

(*b*) Experiments were conducted in the batch soaker–steamer by putting the paddy in hot water, which had been initially heated to a desired temperature, higher than under (*a*). The soaker was then closed at the top by the lid and the paddy allowed to soak. No attempt was made to maintain the temperature of the water at the higher level but the final temperature of the soak water was noted. After soaking for definite periods the paddy was steamed as under (*a*). The moisture and thiamine contents of paddy steeped at different temperatures and for varying periods are shown in Table II.

Table II. The moisture and thiamine contents of paddy after soaking and steaming under particular conditions

No. of obs.	Initial temp. of soak water, °C	Final temp. of soak water, °C	Soaking period, h	Paddy after soaking		Paddy after soaking and steaming	
				% Moisture	Thiamine, μg/g	% Moisture	Thiamine, μg/g
1	100	73	2·0	52·0	2·90	56·9	2·95
2	95	66	2·5	47·5	2·88	52·6	2·75
3	95	63	3·0	51·2	2·78	55·8	2·76
4	90	69	2·0	42·3	2·86	45·3	2·88
5	90	62	2·5	45·1	2·85	49·6	2·83
6	85	60	2·5	40·8	2·75	43·2	2·61

(*ii*) *Continuous soaker–steamer.* Soaking of paddy in a continuous soaker–steamer (Fig. 2, (*a*) and (*b*)) was conducted in the following manner. The soaker trough was filled up with water, so that the paddy to be charged was just under water, and heated to the desired temperature by passing steam through the jacket. The shaft with paddles was rotated at a speed of 4–8 rev/min (with 1° paddle inclination approx.) so that the paddy was being agitated as well as conveyed from the feed end to the discharge end within a predetermined time. Paddy was then fed continuously from the feed hopper at the rate of 80 lb/h inside the soaker trough by the vibrating feeder. Hot water was added as and when required to put the paddy just under water. The required temperature was maintained inside the soaker by passing

steam or hot water as and when required through the jacket. The paddy after being conveyed to the soaker was scooped out into the steaming zone where live steam was passed continuously. It was then conveyed slowly through the steaming zone with the help of the screw conveyor so that the paddy was kept under the steaming operation for 3 min.

Samples of paddy soaked and steamed at different temperatures for varying periods were analysed for moisture and thiamine content as in (*a*) and (*b*). The results are shown in Table III.

Table III. The moisture and thiamine contents of paddy after soaking and steaming under particular conditions

No. of obs.	Temp. in the soaker, °C	Period of soaking, h	Paddy after soaking		Paddy after soaking and steaming	
			% Moisture	Thiamine, μg/g	% Moisture	Thiamine, μg/g
1	75	1·0	41·3	2·72	45·6	2·68
2	75	2·0	48·3	2·65	51·8	2·71
3	75	2·5	52·3	2·66	55·7	2·68
4	75	3·0	56·4	2·70	59·2	2·71
5	80	1·0	44·3	2·55	47·9	2·62
6	80	2·0	50·4	2·81	54·5	2·75
7	80	2·5	56·0	2·62	60·1	2·66
8	80	3·0	59·6	2·73	64·4	2·58

Discussion

The results obtained with the three processes, two in a batch soaker–steamer and one in a continuous soaker–steamer, show that paddy can be soaked properly (retaining 50–55 per cent moisture) at elevated temperatures in the soakers designed and fabricated for the purpose. When the three processes are compared with regard to the amount of water imbibition in the paddy grains the results indicate that the continuous agitating type soaker–steamer gives the best performance. Thus, after 2·5 h and at an average temperature of 75°C, the paddy when soaked in the batch soaker–steamer by methods (*a*) and (*b*) contained moisture to the extent of 48 and 45 per cent respectively, whereas

in the continuous agitating type soaker–steamer, it was 52 per cent.

The two operations in the batch soaker–steamer as well as the operation in the continuous soaker–steamer appear to be industrially feasible, but more time and labour are required for charging, discharging etc. in the batch processes. Treatment in the batch processes can be completed within 2·25–3 h at 75–80°C and it has no adverse effect on the quality of finished rice. The continuous soaker–steamer would however give a larger turn-over and it can be fully mechanized for large-scale production of parboiled rice. The quality of the rice produced by these processes is equally good and the retention of thiamine is also of a similar order.

Both the batch and continuous soaker–steamers give a much greater through-put than the conventional parboiling processes which normally take 2–3 days. They also produce a rice which is cleaner and, as separate experiments have shown, retains about double the thiamine content of parboiled rice obtained in the conventional manner.

Acknowledgments. Our thanks are due to Dr. A. K. Ghosh who was associated with us at an early stage of this investigation. We would also like to express our gratitude to the Indian Council of Medical Research for financing this work.

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Pilot Plant Studies on Parboiling of Rice—II Dehydration

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Summary. A compact dryer for the parboiling of paddy has been described. The use of steam tubes along the circumference of the shell has made high drying rates possible. The rate of water removal is dependent on steam pressure in the tubes. Blowing of pre-heated air through the dryer shell is necessary. The optimum drying conditions for parboiled paddy as observed in the experimental rotary dryer are: (*a*) the steam pressure in the tubes should be about 45 lb/in^2 gauge; (*b*) the feed rate should be 150 lb/h, approximately; (*c*) the rotation should be about 5 rev/min; (*d*) the inclination of the shell should be nearly 2° to the horizontal and (*e*) air at a rate of 100 ft^3/min should be blown parallel to the feed, pre-heated to a temperature of 90°C.

Introduction

The drying of parboiled paddy is generally carried out in the Orient by spreading it out in a layer 1–3 in. deep in the sun on cement or mud plastered yards. In the rainy season drying becomes a prolonged and uncertain process and microbial action on soaked paddy often becomes very pronounced, giving an unpleasant flavour.

The process usually takes 3 to 5 days (from soaking to drying) but sometimes even a fortnight during rainy weather. It was therefore of economic importance to devise mechanical dryers suitable for the purpose.

However, dehydration of paddy unless carried out under well regulated conditions causes development of cracks on the surface of the paddy which results in excessive breakage during milling. It has been reported by several workers that drying can be rapid when the moisture content of the paddy exceeds 35 per cent and

also when it has been reduced to 18 per cent but between these limits drying must be slow and carefully controlled to avoid cracking of grains.[1] It has also been experimentally found that the drying of parboiled paddy is always of a 'falling rate' nature.[2]

After some preliminary investigations an experimental rotary dryer was designed and fabricated for drying parboiled paddy. Results obtained with this dryer are given in this paper.

Equipment

The rotary dryer. The dryer shell, made of mild steel, was 12 in. in diameter and 8 ft long and was of electric arc-welded construction (Fig. 1). The inside of the dryer was provided with

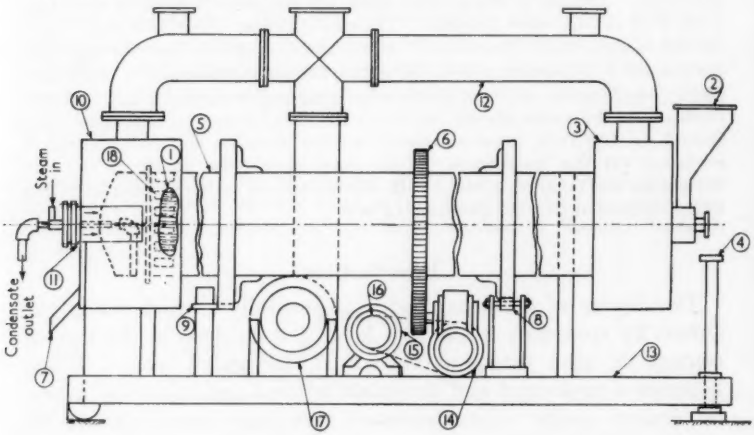

Fig. 1. Rotary dryer for parboiling rice. 1, steam tubes and lifter blades; 2, feed hopper; 3, feed end breeching box; 4, slope adjusting screw; 5, dryer shell; 6, drive pinion; 7, discharge spout; 8, trunion rolls; 9, thrust roll; 10, discharge-end box; 11, stuffing box; 12, air ducting; 13, main base; 14, speed reduction unit; 15, motor for drive; 16, V-pulley; 17, air blower with heater; and 18, paddy outlet

18½-in. B.S.S. longitudinal steam tubes, suitable for 50 lb/in² gauge steam. It was also provided with six 1½ in. × ⅛ in. × 7·5 ft longitudinal flights. The shell was driven by a 1·5-h.p. a.c. motor through a speed reduction gear, variable pitch pulley and V-belt

drive system to vary the dryer rotation approximately from 5 to 20 rev/min in two stages. The dryer was mounted on a frame made of mild steel joists and flats, and the discharge end was fixed on the frame whereas the feed end was supported on a threaded rod so that it could be lifted or lowered to adjust the inclination of the dryer from 0–20° to the horizontal. The feed end breaching box was provided with a feed hopper and a screw feeder to adjust the rate of feed. For circulating hot air through the shell, the dryer was fitted with a blower and a steam heated honeycomb-type air heater. The capacity of the blower was 100 ft^3 of air per min and it was connected to the breaching boxes by a 4-in. duct. A two way valve was fitted in the air duct so that the flow of air could be directed concurrently or counter-currently in the dryer relative to the feed.

Steam inlet and outlet were on the same side of the dryer and passed through a common stuffing box. The condensate outlet was connected to a steam trap.

Experimental procedure. The parboiled paddy (after soaking and steaming) contained 55–60 per cent moisture on the dry basis. The conditions of drying to be fulfilled should be:

(*i*) Reduction of the moisture content from 55–60 per cent to 15–18 per cent (dry basis).

(*ii*) The drying should be uniform.

(*iii*) The grains should not crack during drying and subsequent milling.

(*iv*) The dehydration of paddy should cause no appreciable change in colour or nutritive value compared with sun drying.

The controllable conditions of the experimental rotary dryer were the steam pressure in the tubes, the air rate, feed rate, rotation of the shell, inclination of the dryer and the temperature of the air blown through the dryer.

The inlet and exit air temperatures were measured during drying operations by inserting thermometers in thermometer-wells provided on the two end breaching boxes. Pressure gauges were also fitted to measure the steam pressures inside the tubes and the air heater. The inclination of the dryer and the rotation of the dryer shell were noted before each run. The temperature of the soaked and steamed paddy feed was approximately 60°C when fed in the hopper. The feed rate was regulated and kept

constant by adjusting the sliding plate between feed hopper and screw feeder. Feed rates between 200 and 90 lb of paddy per hour were used. The initial and final moisture contents of the paddy, rev/min of the shell, steam pressure in the tubes, air temperature, feed rate and retention time for paddy inside the shell were noted for each run. The grain temperature at the discharge end was determined by collecting the hot grains just after discharge in a 'thermos' and inserting a thermometer inside the flask. Before paddy was fed into the dryer the steam was turned on in the steam tubes and the air heater, to maintain the desired steam pressure in the tubes and to pre-heat the air to the desired level respectively.

A few test runs were carried out by blowing hot air counter-currently. This resulted in over-heating of the grains and it was also noted that an appreciable amount of air went out of the dryer through the discharge spout without being used for drying. Hence parallel current air was used throughout the study, the air rate being kept constant at 100 ft^3/min. The rotation of the dryer shell was also kept to the lowest limit which was 5 rev/min.

Paddy used for the experiments was of the 'Patnai' variety and was previously heated in the continuous soaker–steamer,[3] soaking being carried out in open steam.

Results and Discussion

Four series of steady-state runs were carried out to study the drying conditions for parboiled paddy. In the first three series, dryer inclination, feed rate and steam pressure in the tubes were varied while the other adjustable conditions were kept constant. In the fourth series, air temperature was varied from 90 to 30°C, keeping other variables constant. The air was blown parallel to the feed at the rate of 100 ft^3/min. Percentage moisture of feed and of the dried product were determined as described previously.[3]

The experimental results obtained in each series and the operating conditions are summarized in Tables, I, II, III and IV.

At constant feed rate and shell rotation, variation of dryer shell inclination to the horizon had a substantial effect on the holding time of paddy in the dryer and consequently on the extent of

Table I. Percentage removal of moisture in relation to dryer inclination. Steam pressure in tubes = 25 lb/in² gauge; pre-heated air temperature = 90°C; shell rotation = 5 rev/min; feed rate = 100 lb/h

Run no.	Dryer inclination, °	Holding time in dryer, min	% Moisture (dry basis)		% Moisture removed (dry basis)
			Feed	Product	
1	10	20	58·2	29·5	28·7
2	5	26	60·1	28·5	31·6
3	2	31	58·6	22·3	36·3
4	0	34	56·9	16·3	40·6

moisture removal from the paddy. On reducing the inclination from 10° to 0° the holding time in the dryer increased by 14 min which also caused an increase in the extent of moisture removal by approximately 12 per cent.

It is evident from the results in Table II that the holding time in the dryer increases as the feed rate decreases and a decrease of

Table II. Percentage removal of moisture in relation to the feed rate. Steam pressure in tubes = 45 lb/in² gauge; pre-heated air temperature = 90°C; dryer inclination = 2°; dryer shell rotation = 5 rev/min

Run no.	Feed rate, lb/h	Holding time in the dryer, min	% Moisture (dry basis)		% Moisture removed (dry basis)
			Feed	Product	
A					
1	200	22	60·4	26·6	38·8
2	150	26	60·1	20·7	39·4
3	100	32	58·2	15·3	42·9
4	90	34	57·7	13·1	44·6
B (Steam pressure in tubes = 35 lb/in² gauge. Other conditions as in (A))					
5	200	22	60·5	30·8	29·7
6	150	26	57·2	20·4	36·8
7	100	32	56·8	16·2	40·6
8	90	34	59·3	16·2	43·1
C (Steam pressure in tubes = 25 lb/in² gauge. Other conditions as in (A))					
9	200	21	60·5	33·7	26·8
10	150	25	60·1	28·2	31·9
11	100	32	58·6	22·3	36·3
12	90	34	60·3	21·8	38·5

feed rate of 50 lb increases the holding time by 4–6 min, thereby simultaneously increasing the percentage moisture removal. It is interesting to note that removal of moisture is not proportional to the holding time. A decrease in feed rate from 200 to 150 lb/h results in an increase in the holding time of 4 min and an increase in moisture removal of 5·6 per cent (Runs 1 and 2) while a decrease in the feed rate from 150 lb/h to 100 lb/h results in an increase in holding time of 6 min and in moisture removal of only 3·5 per cent (Runs 2 and 3). This would indicate a falling rate of drying. It has been observed that if feed rate is kept within 100–150 lb/h, the drying can be accomplished to the desired level in the experimental rotary dryer in about half an hour.

The steam pressure in the tubes had a very significant effect on drying. This is shown by the results in Table III.

Table III. Percentage removal of moisture in relation to steam pressure. Pre-heated air temperature = 90°C; dryer inclination = 2°; dryer shell rotation = 5 rev/min; feed rate = 150 lb/h

Run no.	Steam pressure in tubes, lb/in² gauge	Grain temp., °C	% Moisture (dry basis)		% Moisture removed (dry basis)
			Feed	Product	
1	45	75	60·1	20·7	39·4
2	35	70	57·2	20·4	36·8
3	25	64	60·1	28·2	31·9
4	0	38	55·0	41·2	13·8
5	45	70	58·2	15·3	42·9
6	35	66	56·8	16·2	40·6
7	25	62	58·6	22·3	36·3
8	0	40	54·4	38·6	16·8

When no steam was introduced into the steam tubes the moisture removal from paddy was only 16·8 per cent (Run 8), though pre-heated air at 90°C was blown at a rate of 100 ft³/min. The grain temperature at the discharge end of the dryer decreases with the reduction of steam pressure and for every 10 lb/in² gauge pressure reduction the grain temperature is reduced by about 5°C.

A steam pressure of about 45 lb/in² gauge can be safely employed in the steam tubes with a feed rate of 100–150 lb per hour.

Table IV. Percentage moisture removal in relation to the pre-heated air temperature. Steam pressure in tubes = 35 lb/in² gauge; dryer inclination = 2°; dryer shell rotation = 5 rev/min; feed rate = 150 lb/h

Run no.	Pre-heated air temperature, °C	% Moisture in		% Moisture removed (dry basis)
		Feed	Product	
1	90	57·2	19·5	37·7
2	70	60·1	25·9	34·2
3	50	55·0	22·5	32·5
4	30	60·0	31·2	28·8
5	(No air blown)	58·2	47·1	11·1

The temperature of air blown in has only a small effect on the degree of moisture removal compared with the effect of steam-tube temperature (i.e. steam pressure in steam tubes). A reduction in the air temperature of 20°C decreases the moisture removal by only about 4 per cent, other conditions being kept constant. If however no air is blown the percentage of moisture removed drops to 11·1 (Run 5) which indicates that the water vapour coming out from the paddy as a result of evaporation due to the contact with the hot steam tubes should be blown out from the dryer. Though steam pressure in tubes is the main source for supplying the heat energy for drying, air blowing is essential for an appreciable drying rate in the dryer. The air can be pre-heated to a temperature of about 90° without any damage to the grains.

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Pilot Plant Studies on Parboiling of Rice—III Effect of Hot Soaking and Mechanical Drying on the Nutritive Value of Parboiled Rice

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Summary. Hot soaking of paddy in the parboiling process does not significantly affect the digestibility of rice compared to 'raw' rice. Mechanically dried parboiled rice appears to be slightly more digestible *in vitro* than sun-dried parboiled rice. It is also significantly superior in thiamine value.

Introduction

During the parboiling process, water-soluble nutrients penetrate from the outer layers of the grain into the inner layers. This results, after milling, in a finished product of higher thiamine, riboflavin and niacin content.[1–3] However, since the parboiled rice produced by indigenous methods in India is of poor quality,[4] the conditions and equipment of hot soaking, and drying processes for the production of a better quality of parboiled rice have been described in the preceding papers.[5,6] The present paper describes the effect of these processes, carried out under different conditions, on the nutritive value of parboiled rice, with particular reference to the digestibility *in vitro* of the rice samples produced and their thiamine content.

Materials and Methods

A commercial variety of paddy ('Patnai,' grown in West Bengal) was used in this work. Paddy (100 g) was taken for each set of experiments. The soaking temperature and time were kept

constant and the time of steaming and temperature of drying were varied. An aqueous solution (10 mg/ml) of takadiastase (Park Davis & Co.) was used. One millilitre of the enzyme solution was found to be capable of producing 200 mg of glucose in 1 hour at 30°C, acting on 2 per cent Lintner starch solution.

Wheat β-amylase used in this work was prepared by the method of Ghosh.[7] One millilitre of the enzyme solution was capable of producing 600 mg of maltose per hour at 30°C acting on a 2 per cent Lintner starch solution. Acetate buffer (0·1 M) of pH 4·8 was used in these studies.

Digestibility estimation. The processed rice was powdered and sieved in a 100-mesh sieve. The rice powder (5 g) passing through 100 mesh was made into a paste with a little water and then added to 100 ml of boiling water and the solution was boiled for 2 min and cooled. The cold solution was made up to a definite volume with 0·1 M acetate buffer, pH 4·8. Aliquots were taken in large test tubes and maintained at 30°C. One millilitre of the enzyme solution (takadiastase or wheat β-amylase) was added separately to each of the tubes. After specified intervals of time the reaction was stopped by adding 20 ml of 1 per cent H_2SO_4 solution and the final volume in each tube was made up to 50 ml. Five millilitre portions from each tube were taken for the estimation of reducing sugar by the method of Kneen and Sandstedt.[8] Separate controls were run in each case. The reducing sugars obtained with amylase and takadiastase are reported as maltose and glucose respectively.

Thiamine estimation. The thiamine content of rice was estimated as described previously.[5]

Sun-drying. Where sun-drying of paddy was carried out, the paddy was dried in the open sun in $\frac{1}{2}$-in. thick layers on a concrete floor for two consecutive days from 10 a.m. to 5 p.m. at an ambient temperature of 30–35°C. This is the period necessary for proper sun-drying, though it may be exceeded in the rainy season.

(*a*) *Digestibility of 'raw' and parboiled rice prepared on pilot-plant scale prior to drying.* Table I does not show any significant differences in digestibility between 'raw' rice and that parboiled by soaking at 73–80°C for 2–3 h and steaming for 2–5 min.

(*b*) *Effect of different drying conditions on the digestibility of parboiled rice.* These digestibility tests were carried out mainly

Table I. Digestibility of raw and parboiled rice by takadiastase and β-amylase

Sample no.	Conditions of treatment: Soaking time, h	Soaking temp., °C	Steaming time, min	Reducing sugar formed by 10 mg enzyme after digestion periods of: 15 min As mg glucose by takadiastase	60 min As mg glucose by takadiastase	15 min As mg maltose by β-amylase	60 min As mg maltose by β-amylase
1	('Raw' rice[a])		–	53	125	76	118
2	3	73	2	53	131	88	121
3	3	73	5	59	118	70	103
4	2·5	75	5	64	130	64	130
5	2·0	80	5	64	134	91	112

[a] 'Raw' rice is rice obtained by milling sun-dried paddy without parboiling. The same sample of paddy was parboiled for these experiments.

to note the effect of drying conditions on parboiled rice dried in the sun and in the mechanical rotary dryer described earlier.[6] The digestibility experiments were carried out with wheat β-amylase on samples undergoing different drying treatments. The results are given in Table II.

Table II. Digestibility of hot soaked and mechanically dried rice. The soaking temperature and time were 75–77°C and 2·5 h respectively, the steaming time being 3 min. The parboiled paddy was dried in the experimental dryer under different conditions

Run no.	Drying conditions in rotary dryer: Drying time, min	Steam pressure in tubes, lb/in^2 gauge	Max. grain temperature, °C	Digestibility (mg maltose) formed by 1 mg enzyme in 15 min
1	(Raw rice)			81
2	(Hot soaked and sun-dried[a])			87
3	27	50	77	108
4	27	40	78	120
5	29	35	74	101
6	32	40	86	93
7	33	45	87	93
8	38	40	84	114

[a] Dried in the sun for 2 days.

These results would seem to indicate a slightly beneficial effect on the digestibility of mechanically dried parboiled rice as compared with that of sun-dried parboiled rice.

(*c*) *Effect of different conditions of parboiling and drying processes on the thiamine content of rice.* The thiamine values of mechanically dried rice as well as those of sun-dried rice obtained under different conditions of soaking, steaming and drying were estimated to find whether the drying conditions have any effect on

Table III. Thiamine values of lots 'a' and 'b' of 'Patnai' paddy soaked at 75–77°C for 2·75 h, steamed for 3 min and then dried and milled in a huller. The thiamine values of rice produced by dehusking lots 'a' and 'b' respectively by hand were 3·88 μg/g and 2·80 μg/g

No. of obs.	Max. grain temp. during drying, °C	Steam pressure in tubes, lb/in² gauge	Holding time of paddy in dryer, min	% Moisture of rice (dry basis)	Thiamine content, μg/g
		Lot 'a'			
1	30–35	(hot soaked and sun-dried)		6·98	2·06
2	72	35	15	6·69	3·10
3	71	25	32	9·3	2·25
4	71	25	22	12·3	2·40
		Lot 'b'			
1	30–35	(hot soaked and sun-dried)		11·8	1·75
2	78	40	27	11·9	1·87
3	79	35	33	11·5	1·90
4	83	40	35	12·1	1·80
5	86	40	32	11·9	1·90
6	47	45	33	11·0	1·95
7	73	35	30	11·6	1·95

the retention of thiamine content in finished rice. The differently prepared parboiled paddy samples were milled in a huller under uniform conditions and thiamine was estimated. The results are given in Table III. Two different lots of 'Patnai' variety of paddy, 'a' and 'b', were used.

Table III indicates that mechanical drying was more efficient in retaining thiamine in rice compared with the sun-drying processes. Variation in the drying temperature (70–87°C) in the

mechanical dryer does not appreciably change the thiamine content, if the drying time is kept within certain limits (25–35 min approx.).

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A New Press for the Disruption of Micro-organisms and other Cells*

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Summary. A new press (the X-press) has been developed for the disintegration of cells in the frozen state (mostly at $-25°C$). By forcing the frozen cells contained in a cylinder through a hole much smaller than the diameter of the cylinder but much larger than the size of the cells, a satisfactory disintegration can be obtained.

By forcing the cells through the hole repeatedly rather than once, a higher degree of disintegration can be achieved. By making the receiver identical to the charge chamber, repeated operations are possible without uncoupling the press.

The disintegrated material is very easily recovered—it is simply taken out of the press in the form of a cylinder containing the frozen, disintegrated material which is then easily homogenized.

This principle has been found satisfactory for the disintegration of a large number of cells, *viz.* bacteria, yeasts, moulds, plant and animal cells and, with regard to biochemical questions, it has been found to be superior to existing methods which do not treat the bacteria in the frozen state. The mechanism of the disintegrating action is discussed.

Introduction

A large number of methods have been developed for the purpose of disintegrating cells, most of them dealing with cells in liquid suspensions.[1–4] This means that the cells have to be treated either at a temperature above, or in the neighbourhood of, zero or in a suspension medium other than water. Hughes[5] has, however, developed a different method in which the bacteria are treated in the frozen state in the cylindrical hole of a press, in which the material is forced through a narrow slit. This slit is formed by assembling two metal blocks each having a depression shaped like a cylinder cut in half along the axis. The depressions form a

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complete cylindrical hole with a slit situated on each side of it. Hughes reported very good results with this press but some features in his article, and in our experience with his press, caused us to attempt to develop a different approach to the freeze-pressing disintegration technique.

When uncoupling the Hughes press after use, it was often noticed that a layer of material almost a millimetre thick was located between the two halves of the press, indicating that the narrowness of the slit was not critical for the disintegration effect. It was also noticed that the material which had not passed through the slit and the material which sometimes slipped up around the piston was likewise disintegrated to some extent. When discussing the mechanism of disintegration in his press, Hughes considered the shearing forces in the presence of abrasives, possibly in the form of ice, as being the major cause of disruption. He could also obtain disintegration in a closed tube if he applied pressure sufficiently suddenly. In order to obtain a sudden pressure, a guillotine-fashioned plunger hammer was developed.[6]

In order to test whether the shearing forces in flowing ice are sufficient to disrupt bacteria, the press described below was developed. It has been named the 'X-press', originally because it was a purely experimental press but later also because of the rapidity of disintegration (= express).

Materials and Methods

Description of the Press

The press consists of two main cylindrical parts which are kept together by a threaded ring (Fig. 1). Between the parts is placed an exchangeable disc, the hole or holes of which may be of different sizes. Each main part is provided with a short piston which forces the material through the hole or holes in one or other direction when the unit is compressed by means of a hydraulic jack. In this way, the material may be pressed through the holes many times without uncoupling the press. The two main parts of the large X-press are made from Swedish steel (UHB Stainless 44; Hv 245, Rockwell C 22·6, Brinell 245) which is relatively

soft and which it is not possible to harden. The disc is made of the same material. The pistons are made of tool steel (UHB Arne) which is hardened (Hv 724, Rockwell C 60·5) but they are also more easily oxidized.

The clearance between the pistons and the cylindrical holes is 0·01–0·02 mm. After a number of runs traces of soft steel from the main parts may become attached to the pistons and corresponding furrows will be made in the surface of the holes which will

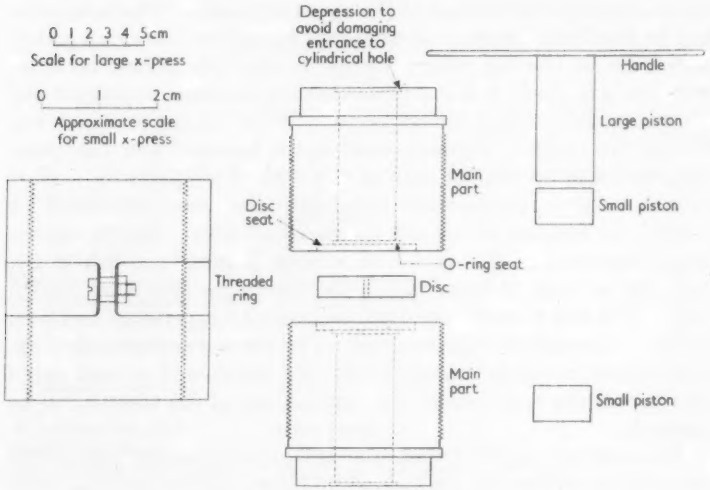


Fig. 1. The X-presses

increase friction. To avoid too strong a friction between pistons and holes, it has been found satisfactory to clean the pistons with emery-cloth. The press, which has now been in use for almost a year, has been run satisfactorily in this way hundreds of times. The small X-press is made completely of hardened steel and therefore no furrows have occurred in the hole. In the small press, the packing between the main parts and the disc is effected by means of thin copper washers and, in the large press, by means of hard rubber O-rings.

Handling of the Press

(The large X-press is chosen as an example.) The press was screwed together by hand and one of the small pistons put into it. The holes were closed with rubber stoppers and the whole press cooled down in an alcohol bath (−60°C) (the pre-cooling temperature is not critical). After 30 min or more, the stoppers were removed and the pistonless end of the unit filled with the material either in the frozen state or in suspension, the latter method of course making the best use of the space available. The hole must not be filled with more than about 25 ml so that the small piston and 2 cm of the big piston are inside the hole before the high pressure is applied; this is a precautionary measure to prevent the big piston from being forced askew. When all the material was frozen, the rubber stoppers were again inserted and the press transferred to an alcohol bath at the desired temperature. Most of the routine, preparative disintegrations were performed at −25°C for reasons which will be discussed later. In the experiments reported in this article, the large X-press was left in the bath for at least 12 hours before the disintegrations were carried out. This was to make sure that the desired temperature had been attained throughout the material. The press was then taken up, the rubber stoppers removed and the pre-cooled second small piston and the large piston were put on top of the material to be treated.

Pressure was applied with the aid of a hydraulic jack. The pressing operation was performed by increasing the pressure until suddenly, at a certain pressure, depending on the hole(s) and the temperature, a loud crack was heard and some material flowed through the hole(s) and the pressure fell. The pressure was then again increased and the procedure repeated until all the material had been forced through the hole. In those cases where it was considered desirable to force the material through the hole(s) of the disc many times, the large piston was moved to the hole on the other side of the press which was turned upside-down. After the pressing operation, the press was uncoupled using a pair of plumbing pliers and a wrench. The treated material was easily removed in the form of a solid cylinder. The press was then washed in hot water and dried quickly. It has not been found

necessary to grease the surfaces. When the surfaces of the pistons became oxidized or contaminated with soft steel from the main parts of the press, they were easily cleaned by means of an emery cloth.

In most of the experiments with the small X-press the apparatus was pre-cooled for 30 min at −60°C. The bacterial suspension (2 ml) was then poured in and the press left for another half hour at −60°C before the disintegration was carried out using the same hydraulic jack.

Cultivation of Test Organisms

Table I

Micro-organism	Cultivation medium	Ref.	Temp., °C
Escherichia coli B	Hook	7	37
	Difco broth		37
Proteus mirabilis	Difco broth		37
Staphylococcus aureus 209	Difco broth		37
Streptococcus faecalis	Difco broth		37
Streptococcus pyogenes S84	Ox-heart infusion		37
Leuconostoc mesenteroides	Semisynthetic		23
Bacillus megaterium	2% peptone broth with pulse aeration	8	30
Clostridium butyricum	Maize flour broth		37
	Difco AC-broth		37
Mycobacterium phlei	Difco broth		37
Bordetella pertussis	Cultivated on a large scale	9	37
Saccharomyces cerevisiae	Bought as press yeast		

All cultivations of aerobic bacteria with culture volumes of less than 5 l. were run on a rotary shaker, often with vigorous sparger aeration. When the cultivation was concluded, the bacteria were cooled in an ice-water bath and spun down in a refrigerated centrifuge. The centrifugates of *B. megaterium* and *L. bulgaricus* described in Tables III–VI were washed and suspended in citrate–phosphate buffer, pH = 7·4 (65 ml 0·1 M citric acid, 500 ml 0·1 M sodium citrate, 436 ml 0·2 M $NaHPO_4$ and 1 l. Aq. dest.), dispensed in test tubes, frozen and kept at −25°C until used. The contents of one tube were then thawed and placed in the pre-cooled small X-press or diluted 1 : 10 in citrate–phosphate buffer

and placed in the large X-press, and the material was allowed to freeze. In one series of experiments (Table V), where the nitrogen content of the supernatants was investigated, the bacteria (*B. megaterium*) had been frozen in Petri dishes and were thawed and treated in the large X-press without any preceding dilution. After the pressing operation, all materials were thawed, diluted in citrate–phosphate buffer and put in an ice-water bath for 2 or 3 hours (different times in different experimental series). These times were arbitrarily chosen to allow time for the soluble part of the material and its sedimentable portion to attain equilibrium.

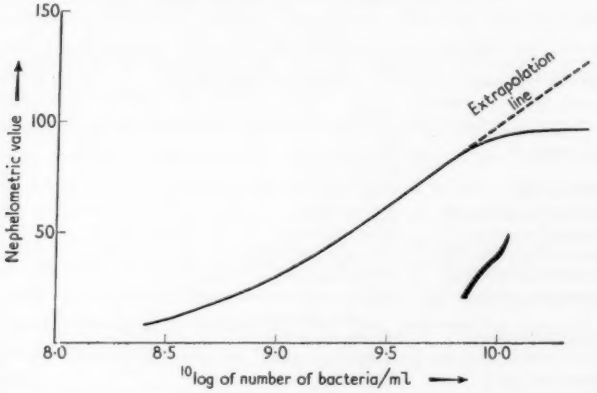


Fig. 2. Relationship between logarithmic number of bacteria (*E. coli* B)/ml and nephelometric value. A similar curve was obtained with *Staphylococcus aureus* 209

Citrate was used in the buffer in order to avoid enzymatic action due to microbial desoxyribonuclease since citrate binds the Mg-ions necessary for the action of known desoxyribonucleases of microbial origin.[10]

The disintegration effect was estimated as: (*a*) the turbidity in a nephelometer (B.Lange, Berlin) having its linear semilogarithmic scale range (Fig. 2) at about 20–85 units (by choosing appropriate dilutions, the turbidity can be estimated over a wide range); (*b*) the extinction of the supernatants at 2,600 and 2,800 Å in a Beckman DU spectrophotometer after centrifuging for 15 min at

24,000*g* in a refrigerated centrifuge (International or Servall); and (*c*) the degree of absence of distinctly stained cells after staining with diluted fuchsin (Gram-negative bacteria), Gram's stain (Gram-positive cells) or Hallberg's Nachtblau stain[11] (*Mycobacterium phlei*). The disintegration was estimated as complete (+ + + + + +), 90–99 per cent (+ + + + +), 70–90 per cent (+ + + +), 40–70 per cent (+ + +), 10–40 per cent (+ +) and absent (−). In a few instances, the disintegration was also estimated by means of a nitrogen determination (Kjeldahl) on the supernatants after centrifuging for 30 min at 30,000*g*.

None of these ways of estimating the disintegration effect is sufficient alone. The estimation of the microscopical smears is rather rough and so also are the other methods of estimation. The extinction of the supernatants can only be relied upon to show very great differences (e.g. pressed and untreated material), probably because part of the pellet will be resuspended when pouring off the supernatant, though no turbidity is seen. The nitrogen determinations were made on supernatants from suspensions with higher nitrogen contents which were centrifuged for 30 min at 30,000*g*. It has, later, been observed that, when centrifuging several identical samples of a suspension with a high nitrogen content, the supernatants give more consistent extinction values than when centrifuging the same suspension after dilution. This is probably due to the fact that the centrifugate is firmer when it is large, and also to the fact that the part of the centrifugate which is suspended when pouring off the supernatant must be diluted in order to be comparable to the originally dilute suspensions.

The intensity of light scattered by small particles in dilute suspensions is expressed by Lord Rayleigh[12] as

$$\frac{I_S}{I_0} = \frac{n_1^2 - n^2}{n^2} \frac{NV^2}{\lambda^4 r^2} (1 + \cos^2 \beta)$$

(I_S = intensity of scattered light; I_0 = intensity of incident light; n = refractive index of the medium; n_1 = refractive index of the particles; N = number of particles; V = volume of each particle; λ = wave-length of incident light; β = angle between the scattered and incident beams.) The particles are supposed to be contained in such a small volume that the vectors r, joining

the individual particles and the point of measurement, can be considered equal.

When the micro-organisms are disintegrated, the cell wall is broken up and the cell content released. This causes a reduction in refractive index of the cells (Fig. 8) and consequently also a reduction in the scattered light. It is not improbable that the breakdown of cell walls proceeds further causing an additional reduction in turbidity since turbidity is proportional to the square of the individual volume of the particles but directly proportional to their number.

Results

The first experiments were made with the small X-press and the experimental conditions were made to approach as closely as possible those of Hughes' press in that discs with slits of equal length (8 mm) but of various widths were used (Fig. 3). However,

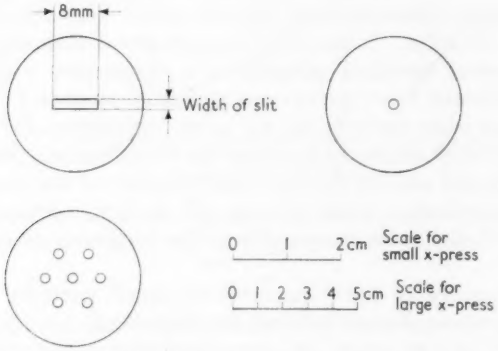


Fig. 3. Discs with different numbers, sizes and shapes of hole(s)

a round hole was also used in some cases to test whether the shape of the aperture was of importance. Frozen centrifugates of *E. coli* B were used as test material. They were cut up but not thawed, and consequently the whole space of the press could not be exploited. The total mass treated may obviously have differed somewhat from time to time. The disintegration effects

in these cases were estimated by suspending all the materials for the same length of time in equal volumes (15 ml) of 0·1 M sodium phosphate, pH = 7·0, at 2°C and estimating the nitrogen content of the supernatant by the Kjeldahl method (Table II). From

Table II. *E. coli* B frozen, stored for ½ year, cut up and pressed in the small X-press with discs of different sizes and hole-shapes (Fig. 3). Pressed once with the aid of a fly press

A Width of the slit, mm	B Area of the hole, mm^2	C Wet weight of bacteria, g	D Nitrogen in supernatant, mg/ml	D/C mg N/ml/g bact.
0·4	3·2	0·703	1·00	1·4
0·5	4·0	0·842	1·33	1·6
0·6	4·8	0·900	1·23	1·4
0·7	5·6	1·173	1·42	1·2
0·8	6·4	0·962	1·42	1·5
0·9	7·2	1·336	1·72	1·3
1·0	8·0	1·128	1·32	1·2
One round hole (2·4 mm diam.)	4·5	1·144	1·60	1·4
Seven round holes (each 0·9 mm diam.)	4·5	0·956	1·20	1·3
Control, just thawed		1·637	0·26	0·16

this table, it can be seen that the dissolved nitrogen did not differ markedly when discs having slits of different widths were used. No marked changes in the disintegration were noted when the hole(s) was circular, nor did the area of the hole(s) cause any alterations within the ranges investigated. A similar experiment was performed in which the influence on the disintegration effect caused by different rates of freezing was investigated (Table III). Freshly harvested and washed *E. coli* B cells were frozen at different temperatures in order to obtain ice crystals of different sizes and shapes. The disintegration obtained with the different samples did not differ markedly, either when estimated as mg soluble nitrogen per ml obtained per gram of wet weight bacteria or when determined as percentage soluble nitrogen.

Table III. *E. coli* B, freshly cultivated, washed and frozen down in different ways, and then packed in the small X-press without thawing. A disc with one hole of 2·5 mm diam. and a hydraulic press have been used. Passed through the hole once

A Mode of freezing	C Wet weight of bacteria, g	E Nitrogen in total suspension, mg/ml	D Nitrogen in supernatant, mg/ml	D/C mg N/ml/g bact.	D/E × 100 Percentage of dissolved nitrogen
On aluminium foil on solid CO_2+alcohol	1·024	1·58	1·07	1·05	68
In air, −24°C	1·369	2·05	1·41	1·03	69
In air, −20°C	1·182	2·00	1·26	1·07	63
In air, −10°C	1·026	1·71	0·99	0·96	58

When pressing *B. megaterium* many times through the hole(s) an increased disintegration was obtained as illustrated in Tables IV and V and Fig. 4 by means of nephelometric values, microscopic observations (Fig. 6) and nitrogen in supernatants. The increase in the nitrogen content of the supernatant corresponds

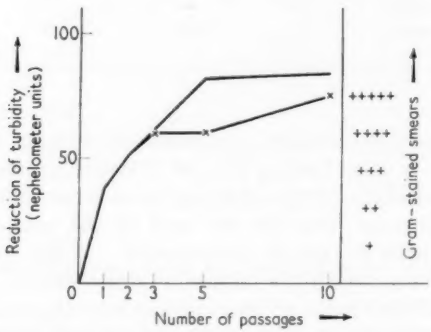


Fig. 4. Disintegration effect of various numbers of passages through the hole (2·5 mm) of the disc. Turbidity estimated by nephelometer. (——— turbidity; x——x Gram-stained smear). The turbidity of the untreated sample, whose 1:10 dilution value was not situated within the linear range of the nephelometer scale, was calculated from the 1:100 dilution by extrapolation (Fig. 2). This turbidity should be 128 in order to be comparable to the other 1:10 values. Compare Table IV and Fig. 6

Table IV. *B. megaterium* ($8{\cdot}2 \times 10^9$ bact./ml) treated in the small X-press (2 ml) with a disc with one hole of 2·5 mm diam. The bacteria have been frozen in different ways, forced through the hole a varying number of times and at different temperatures. Nephelometric values above 90 and below 25 not used in the diagram (Fig. 4)

Mode of freezing	Number of passages	Temperature at pressing, °C	Nephelometric values Dilution		Microscopic observation (Gram's stain)
			1:10	1:100	
In pre-cooled press, −60°C	1	−60	90	27	+ +
D:o	1	−60	90	23	+ + +
D:o	2	−60	78	15	+ + + +
D:o	2	−60	73	14	+ + +
D:o	3	−60	73	13	+ + + +
D:o	3	−60	71	9	+ + + +
D:o	3	−60	68	10	+ + + +
D:o	3	−60	62	12	+ + + +
D:o	3	−60	58	9	
D:o	5	−60	46	13	+ + + +
D:o	5	−60	46	7	
D:o	10	−60	48	10	+ + + + +
D:o	10	−60	40	4	+ + + + +
D:o	3	−25	91	28	+ + +
D:o	3	−25	84	22	+ + +
D:o	3	−10	89	25	+ + +
D:o (Control)	0		96	57	—
Control, without freezing	0		96	54	—
D:o	0		96	51	—
In brass tube, −10°C in air	3	−60	59	12	+ + + +
In brass tube, −20°C in air	3	−60	60	18	+ + + + +

to the disintegration of the cells and also to the decrease in turbidity, but the change in extinction at 260 and 280 mμ does not correspond very well to these values. The mode of freezing does not seem to influence the disintegration of *B. megaterium*.

In the large X-press, a few experiments were performed to investigate the influence of the number and size of the holes in the disc. *B. megaterium* was chosen as test organism because previous experiments involving the exposure of this bacterium to

Table V. *B. megaterium* ($8{\cdot}2 \times 10^9$ bact./ml) treated in the large X-press (20 ml) with a disc with one hole of 2·5 mm diam. The bacteria have been forced through the hole a varying number of times. In the last experiment, the bacteria had been stored in tubes, and in the first three experiments, in Petri dishes

Number of passages	Temperature at pressing, °C	Nephelometric values Dilution		Supernatant Extinction after dilution 1:100		Nitrogen, mg/ml	Microscopic observation (Gram's stain)
		1:10	1:100	2,600Å	2,800 Å		
1	−25	94	35	0·51	0·18	1·33	+++
2	−25	75	14	0·61	0·27	1·54	++++
3	−25	64	10	0·59	0·24	1·83	++++
(3)	−60	76	16	0·30	0·12	1·60	++++

several passages through the hole had shown that the nephelometric determinations were suitable for estimating the disintegration effect. The values are shown in Table VI and Fig. 5. It can be seen that increasing the size of the hole(s) in the disc reduces not only the pressures required to cause flow but also the disintegration. An increase in the number of holes increases the pressure drop.

Table VI. *B. megaterium* ($8{\cdot}2 \times 10^8$ bact./ml) treated in the large X-press (20 ml) with discs with varying numbers of holes and hole sizes. The bacteria have been forced through the holes three times in each experiment

Number of holes	Diam. of hole(s), mm	Temp., °C	Nephelometric values Undiluted	1:10	Gram's stain	Pressure, tons/cm² Maximum	Minimum
1	1·5	−25·5	80	17	+++++	1·5–2·5	1·0–1·3
1	2·5	−25	70	11	++++	1·6–1·9	1·0–1·3
1	3·8	−25	80	17	++++	1·4–2·0	0·7–1·3
1	5	−26	79	16	+++	1–1·9	0·5–0·8
1	7·5	−25	92	30	++	0·6–1·3	0–0·9
1	10	−25	95	45	+	0·7–1·1	0·5
4	2·5	−25·5	73	16	++++	1·4–2·1	0·5–0·9
7	2·5	−25·5	73	22	++++	1·5–2·0	0–1·0
7	3·8	−25	80	16	++++	1·3–1·9	0
7	5	−25	93	33	++	0·8–1·3	0(−0·6)
0	—	−25·5	96	53	—	2·5	(0)
1	2·5	−10	73	12	++++	1·0–1·1	0·8–1·0
Control	—	—	96	53	—	—	—

The flow of the material through the hole(s) is accompanied by cracks which are particularly loud when the rapid pressure drop is high, i.e. when using many holes. When using large holes (5–10 mm), the material passes through the hole(s) either with a loud crack accompanied by a considerable pressure drop or with only a soft creaking or rubbing sound accompanied by a very small pressure change. However, a rapid increase in pressure promotes the production of loud cracks and pronounced pressure changes.

The pressure required to force the frozen bacteria through the hole(s) is not always constant during the same pressing operation.

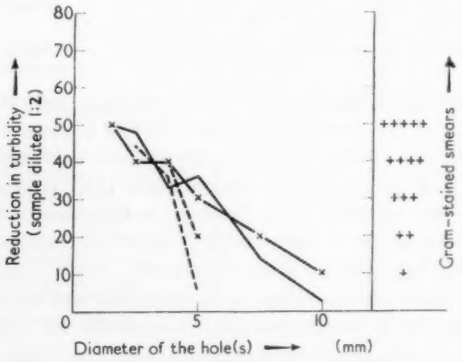


Fig. 5. Effect of various numbers and sizes of holes in the disc (——— one hole, turbidity reduction; x——x one hole, Gram-stained smears; – – – seven holes, turbidity reduction; x– – –x seven holes, Gram-stained smears)

A higher pressure is required at the beginning of a pressing operation. This is particularly evident when the material has already passed through the hole(s) one or more times.

The influence of the temperature on the disintegration effect was studied in both X-presses. With the large X-press, the pressures required were also measured but no reliable pressure measurements could be made with the small X-press. In the small press an increase in temperature above $-25°C$ seemed to reduce the disintegration effect. With the large X-press, however, it was noted that the pressure required to cause the material to flow

Table VII. *Lactobacillus bulgaricus* treated in the large X-press at different temperatures with different numbers of holes in the disc. The bacteria have been forced through the hole(s) three times in each experiment

Number of holes	Diam. of hole(s), mm	Temp., °C	Nephelometric values	Gram's stain	Pressure, tons/cm²	
					Maximum	Minimum
1	2·5	−25	30	++++	1·3–1·9	0·8–1·3
1	2·5	−16	28	++++	0·8–1·3	0·5–1·0
1	2·5	−14	29	+++	0·8–1·3	0·6–1·0
1	2·5	−8	34	+++	0·6–1·1	0·5–0·8
7	2·5	−23	36	++++	1·3–1·4	0·4–0·8
7	2·5	−10	43	+++	0·9	0·5–0·6
7	2·5	−8	43	+++	0·8–0·9	(*a*) 0·4–0·5 (*b*) 0·8
Control	—	−60	94	−		

could be reduced without any accompanying reduction in the disintegration of the micro-organisms (Tables VI and VII).

Many different species of micro-organisms were tested in the X-presses. For this series of experiments the presses were provided with one circular hole having a diameter of 2·5 mm. Table VIII shows that a wide range of micro-organisms and other cells are disintegrated in the X-presses. Light- and electron microscopic observations (Figs. 6–9) show cell wall disruptions for various cells with accompanying liberation of cell content. Some kinds of cells are more easily disintegrated than others, the results obtained in this respect corresponding fairly well to those of Hughes.[5]

A few kinds of cells have been disintegrated in the large X-press for the purpose of isolating cellular substances. The micro-organisms were then treated as cell paste after centrifugation and the animal cells as rather large pieces of frozen tissue. The results are very encouraging. Besides the fact that cells were well disrupted and the tissue completely homogenized without thawing, good physiological and biochemical properties were observed in the disintegrated material in co-operation with other investigators.

'Slow reacting substance (SRS)', found earlier in connection with histamine liberation, has been extracted from mast cell tumours in dogs under circumstances which suggest that the SRS is formed enzymatically.[13]

Table VIII. Disintegration of various kinds of micro-organisms and pollen. The nondisintegrated Gram-positive cells were bluish coloured against a heavily red-stained background. Gram-negative well-stained cells were also easily distinguished from the weaker stained background. (Kind of press: L = large, S = small X-press. Kind of material treated: P = paste, S = suspension, T = tissue)

	Kind of press	Number of passages	Temp., °C	Microscopic observation	Kind of material
Azotobacter vinelandii	L	3	−25	++++	S
Bordetella pertussis	L	3	−22	+++++	P
Clostridium butyricum	L	3	−60	++++	S
Diplococcus pheumoniae	L	2	−37	+++++	P
Escherichia coli B	L,S	3	−25, −60	+++++	P,S
Gibberella fujikuroi	L	5	−25	++++	S
Lactobacillus bulgaricus	L,S	3	−25, −60	++++	P,S
Leuconostoc mesenteroides	S	5	−60	++++	S
Mycobacterium phlei	S	5	−60	+++	S
Proteus mirabilis	L	3	−37	++++	P
Rhodospirillum rubrum	L	3	−37	+++++	S
Salmonella typhimurium	L	3	−25	+++++	P
Staphylococcus albus	L	2	−25	+++	P
Staphylococcus aureus 209	S	3	−60	+++	S
Streptococcus faecalis	S	5	−60	++++	S
Streptococcus pyogenes S 84	L	3	−25	+++	P
Cryptococcus terricolus	L	5	−25	+++++	P
Saccharomyces cerevisiae	L,S	3	−25, −60	+++(+)	P,S
Ragweed pollen	L	2	−60	++++	P
Animal cells	L	3	−25	+++++	T

A 'particulate-free' extract containing an enzyme capable of forming deoxycytidine 5′-phosphate from cytidine 5′-phosphate has been prepared from *E. coli* B.[14]

Atoxic immunogenic substance(s) of *Bordetella pertussis* have been localized to the cell wall. The large X-press makes possible a commercial application of this observation for the preparation of pertussis vaccines.[15]

Lipids have been quantitatively prepared from *Cryptococcus terricolus* for the gas-chromatographic analysis of fatty acids.[16]

Desoxyribonucleic acid has been isolated from liver, thymus and pancreas, and the red blood cells of hens, and purified by zone-electrophoresis.[17]

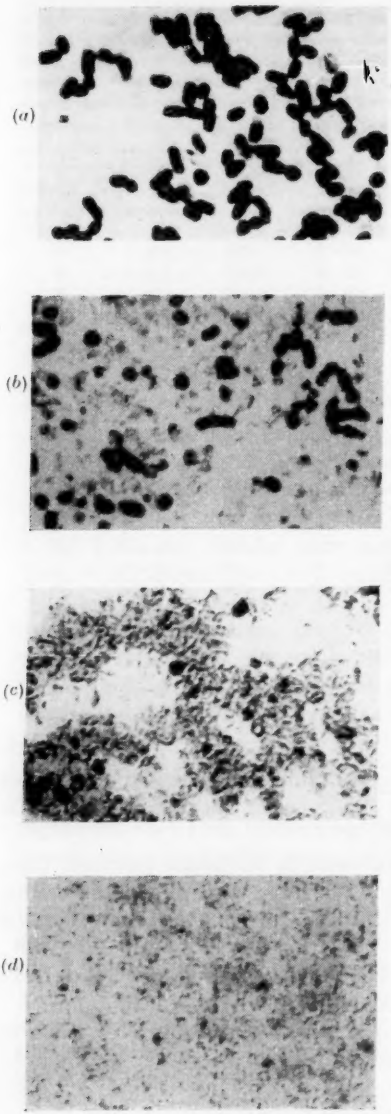
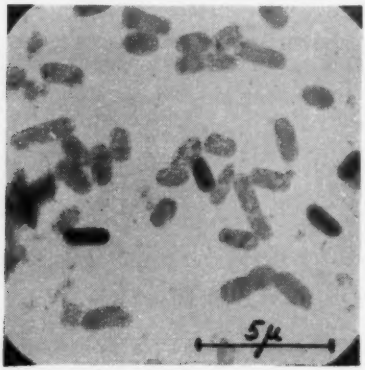
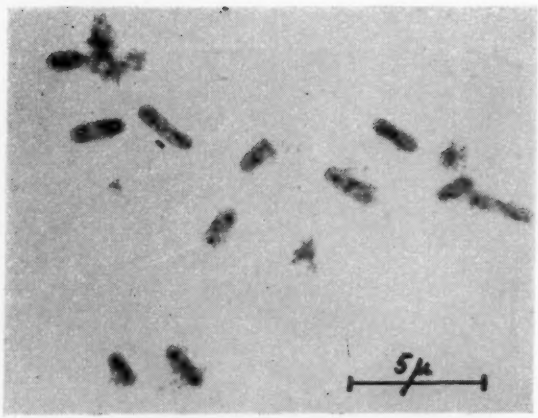


Fig. 6. *B. megaterium* frozen and treated at −60°C in the small X-press with a disc with one hole of 2·5 mm (Table IV). Gram-stained smears. Magnification about 2,000×. (*a*) No pressing (control). Forced: (*b*) once, (*c*) five times and (*d*) ten times through the hole

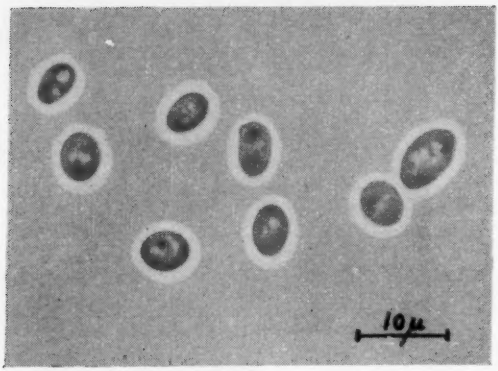


(*a*)



(*b*)

Fig. 7. (*a*) Electron micrograph of *E. coli* B passed once through a 2·5-mm hole in the small X-press. The photograph mainly consists of empty cell walls. A few dark whole cells are also seen. (*b*) *E. coli* B forced three times through a 2·5-mm hole in the small X-press (electron micrograph). The photograph shows cell walls with uneven contours, either due to adsorption of cytoplasmic material or to disintegration of the cell wall



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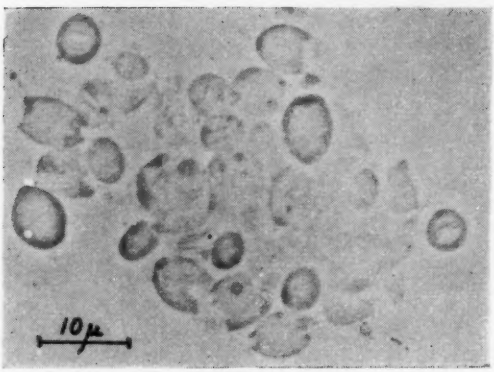
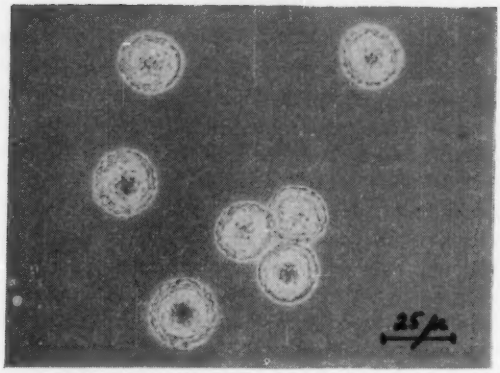


(*b*)

Fig. 8. (*a*) Untreated yeast cells. (*b*) Yeast cells pressed three times (large X-press, 2·5-mm hole). Most cells disrupted with evident damage to cell walls (phase contrast microscopy)



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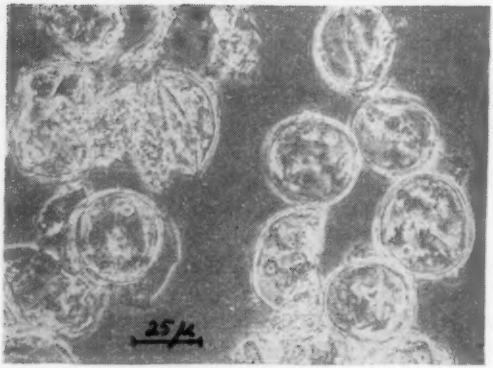

(*b*)

Fig. 9. (*a*) Untreated ragweed pollen. (*b*) Pressed pollen. Note the larger size of these pollens compared with the untreated ones and the damage to the cell walls (phase contrast)

Discussion

Concerning the small holes of the disc described in the early experiments (Tables II and III), the size and shape did not influence the disintegration effect significantly and neither did the mode of freezing. The yield of soluble nitrogen was about the same in all the experiments, being somewhat lower when using washed bacteria (Table III). In the latter case, the dissolved material amounted to about 65 per cent as estimated from the nitrogen content. In these early experiments, the disintegration obtained was however not so good as in later ones if microscopical observations are taken as the basis for judgement. The disintegration also varied somewhat from experiment to experiment, one of the probable causes being the unequal packing of the cylindrical cavity of the press with cubes of frozen bacteria. Salton[18] estimated that the cell wall of Gram-negative bacteria accounts for less than 20 per cent of the cell yield while more recent investigations[19] estimated this figure to be between 20 and 30 per cent. This indicates that if the relative nitrogen contents of the whole cell and the cell wall are similar (i.e. if the dry weight and nitrogen determinations agree fairly well) then this would indicate that the disintegrations in our case are not quite complete. However, the adsorption of cytoplasmic material on cell walls also assists in decreasing the amount of soluble nitrogen. In a future investigation on different disintegration methods and methods for preparing cell walls, a more precise determination of the cell wall of different bacteria is planned.

The disintegration of *B. megaterium* is increased upon repeated forcing of the bacteria through the hole, as judged by nephelometric values, microscopic observations and nitrogen content of the supernatant (Tables IV and V and Figs. 4 and 6). However, the extinction of the supernatant at 2,600 and 2,800 Å does not seem to be a good index of disintegration. Such disparities were also obtained in many other experiments of the same kind and that is why these data are omitted here. The nature of this irregularity is not clearly understood. At present the most probable explanation seems to be that the error of the method is so large that the small relative difference between the cell contents released at high disintegration effects cannot be calculated by

extinction of the supernatant after centrifuging relatively dilute suspensions at 24,000*g* for 15 min. The estimation of the disintegration by means of nephelometric values in which the residual intact bacteria (and, to a smaller extent, the cell walls) are measured is, on the other hand, favoured by a large disintegration because the relative difference between two values with the same absolute difference will be larger at high disintegration effects.

The influence of the size and number of the holes on the disintegration in the X-press is shown by Table VI and Fig. 5. When

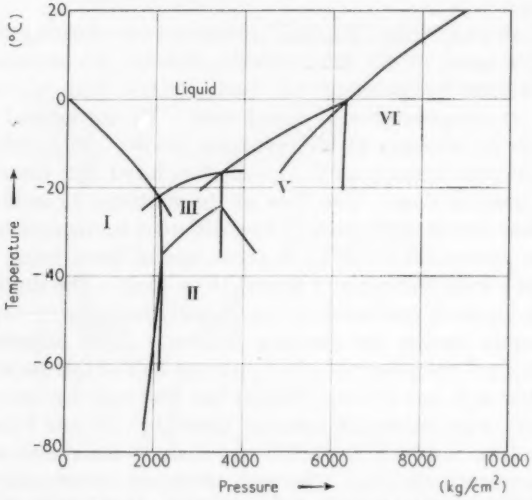

Fig. 10. A part of the phase diagram of water in the liquid–solid region. Redrawn from P. W. Bridgman, *Proc. Amer. Acad. Arts Sci.*, **47**, 439 (1912)

using the large X-press with one hole in the disc, the disintegration is decreased by increasing the diameter of the hole and so is the pressure required to cause the material to flow through the hole. Above 5 mm, these reductions are particularly marked. When using seven holes, the disintegration is most notably decreased when the diameters of the holes exceed 3·8 mm. Using a disc with no hole and increasing the pressure to 2·5 tons/cm^2, which causes simple changes in the crystal structure of the ice from

phase I to phase III (Fig. 10) without flow of the suspended cells (*B. megaterium*) through an orifice, gave no measurable disintegration. This is remarkable considering the disintegration previously obtained with *E. coli* B [20] but *B. megaterium* is known to be more resistant to disintegration than *E. coli* B. With the present experimental equipment it has not been possible to study the influence on disintegration resulting from the rate of increase in the pressure.

The mechanism of the disintegration obtained in the X-presses is not yet quite clear, the explanation following three lines of reasoning:

(*a*) At an early stage, Hughes [5] suspected the shearing forces to be a major cause of the disintegration because he obtained good disintegrations by pressing cell paste mixed with pyrex glass abrasive at temperatures of about zero. He considered the ice to serve as an abrasive at temperatures below $-20°C$, which is a good argument because, as Bridgman [21] pointed out, the strength of ice is greater then. The flow of the material as indicated in Fig. 12 was revealed by casting two different horizontal layers of ice in the pre-cooled ($-60°C$) X-press, one of them being stained heavily red with Saffranin O (Gurr) (Fig. 11a). The dome shape of the ice-layers is produced by the radially converging expansion of the liquid during the freezing process. After adjusting the temperature of the press to $-25°C$, almost half of the material was pressed through one 2·5-mm hole of the disc and the ice cylinder left behind was taken out and cut through. It was found that the boundary between clear ice and stained ice remained well-defined and smooth (Fig. 11b) and, moreover, dome-shaped. A similar observation was made when using a disc either with seven holes of 2·5-mm diameter or with one 10-mm hole. In one single case, the observation indicated in Fig. 11c was obtained. In order to avoid an increase in temperature and, as a consequence, a thawing of the ice at its surfaces of contact with the press, the press was cooled for 1–3 min at $-60°C$ immediately before the pressing operation. However, in these cases also, the same sort of observation was made. All these facts point to a horizontal flow of the material near the disc which might favour shearing forces. When using a disc with one 10-mm hole, a small cavity facing the hole is formed in the ice that has not been pressed

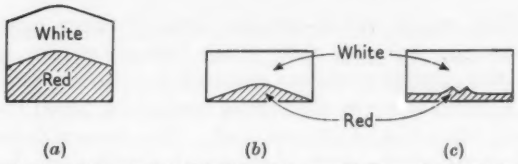

Fig. 11. Section through ice cylinder not pressed through the hole of the disc

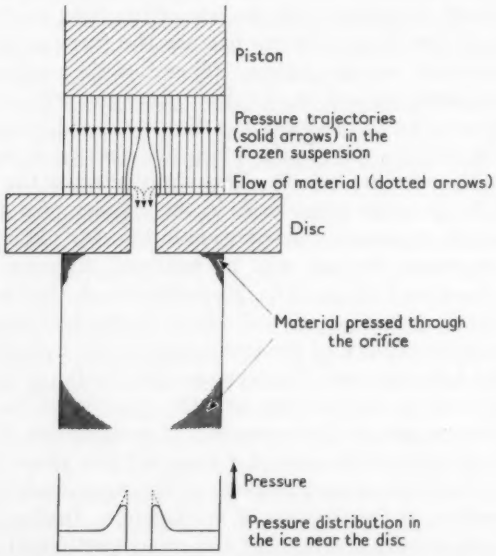
Piston
Pressure trajectories (solid arrows) in the frozen suspension
Flow of material (dotted arrows)
Disc
Material pressed through the orifice
Pressure
Pressure distribution in the ice near the disc

Fig. 12. Pressure and flow in the X-presses. Pressure trajectories of the ice indicated by solid arrows. Direction of flow, when the material passes through the hole in the disc, indicated by dotted lines and arrows. At the bottom of the drawing, an approximate diagram of the pressure distribution is shown. Because of a slight flow of the ice in a narrow region close to the hole, the very high peak of the curve (broken line) will probably not be obtained. The pressure on the wall of the cylindrical cavity is, for the sake of simplicity, not shown in the figure

through the hole. Smaller cavities are formed when using smaller holes.

(*b*) The phase transition of ice I (ordinary ice) to ice III occurs at a pressure of 2·1 tons/cm^2 between -22°C and -35°C and then proceeds with a volume decrease of about 0·2 cm^3/g or 20 per cent (calculated according to Bridgman[3]). The pressure values listed

in Table VII, which are about the same as those required for phase transition, indicate that phase transitions are involved. However, the applied pressures required for the material to flow are often somewhat lower than those needed for phase transition, particularly when large holes are used. This feature is elucidated in Fig. 12 in which the state of the ice in equilibrium, before the material has started to flow appreciably and before any crack has been heard, is shown. At the site of the hole, no reaction is exerted on the ice by the disc to balance the pressure applied by the hydraulic jack *via* the pistons. Therefore the pressure, which should have been applied on the disc where the hole is situated, will be taken up by the parts of the disc in the neighbourhood of the hole (Fig. 12) in a way similar to the distribution of the stresses in a plate with a circular hole.[22] This is indicated by the increased density of the pressure trajectories in the vicinity of the hole and by the diagram of pressure distribution in Fig. 12. It is supposed that this pressure increase will be sufficient to cause a phase transition from ice I to ice III. Probably the dotted top of the pressure distribution diagram will never be reached because of a small inconspicuous flow of the ice nearest to the hole of the disc. When larger holes are used, the pressure distribution in the vicinity of the hole will be still higher and the density of the pressure trajectories correspondingly increased. Consequently, the *applied* pressure does not need to reach 2·1 tons/cm^2 (the phase transition pressure) in order to cause a pressure of this magnitude and thus a phase transition in the vicinity of the hole(s). During the phase transition the tensile strength of the ice is particularly low and this facilitates the flow of the material. Later, when the material has passed through the hole, the ice must revert from ice III to ice I. It is not unlikely that this phase transition causes a great deal of the disintegration because it proceeds with a sudden volume increase of almost 20 per cent. It is probably also the cause of the loud cracks. When studying the material which has passed through the hole, it is often found as a coarse powder at the edges of the cylindrical cavity, giving the impression of an explosive disintegration of the ejected material.

(*c*) The pressure gradient which is caused by the applied pressure and the flow of the material must cause tremendous stresses in the cells which will in turn influenee the disintegration. The

importance of the pressure gradient is also supported by Hughes' observation that disintegration in a tube without outflow was only obtained if the pressure was applied suddenly. The pressure gradients obtained in the X-presses are thought to be greater than those of Hughes' press because of the relatively free outflow of the material in the X-presses. However, phase transitions probably also occur in Hughes' press.

Probably all of the three mechanisms suggested participate in the disintegration of the cells in the X-presses. In addition, they are not clearly distinguished from each other.

When using seven holes in the disc, the pressure drop is greater than when using one hole. An explanation of this would be that, when the pressure is increased sufficiently highly, each hole in the disc will serve as a phase transition nucleus. The material will then flow almost explosively and, since there are many holes, more material will flow through, before the pressure fall alters the crystal structure of the ice, and the pressure fall will consequently be greater. This greater pressure drop, when using a larger number of holes, and the discontinuous flow in the X-presses are facilitated by the inertia of the ice and the hydraulic jack.

A higher pressure is required to cause the material to flow in the beginning of subsequent treatments—this might be due to the absence of a regular crystal structure in the bacteria–ice powder suspension. The subsequent flows, on the other hand, proceed at the same pressure as those of ordinary ice (ice I).

The influence of temperature on the disintegration process is shown in Table VII and to a certain extent in Table IV, using *L. bulgaricus* and *B. megaterium*, respectively, as test organisms. In Table VII, it is seen that when the temperature is increased above $-25°C$ the disintegration in the large X-press is not appreciably decreased when using one hole and only slightly decreased when using seven holes. Above $-22°C$, the phase transition occurs from ice I to liquid and *vice versa*, and the pressure gradients are changed as indicated by the pressure required to cause flow and the pressure obtained after the flow of the material. When using the small X-press (Table IV), greater disintegrations were obtained at $-60°C$ than at $-25°C$ and $-10°C$. At $-60°C$, a phase I–II transition occurs which might have increased the disintegration. The disintegration produced

when using seven holes seems to be slightly reduced when increasing the temperature above $-23°C$ (Table V). The decreased pressures required at higher temperatures might be of particular importance when developing this method for larger masses. Some promising results have already been obtained along this line of investigation. It is also expected that a more precise knowledge of the pressure gradients might explain the experimental results hitherto obtained and become a guide for further development.

When operating the large X-press at $-60°C$, pressures reaching almost 5 tons/cm^2 were applied without causing the material to flow. If the material was left at about 3 tons/cm^2 for about 5 min a sudden crack was heard as the material flowed through the hole and the pressure fell. This increased difficulty of flow at low temperatures must be due to the decreased velocity of phase transitions occurring at low temperatures. It cannot be stated with certainty whether the flow was caused by the time factor or by the increase in temperature but the long time also needed for the subsequent flow to occur speaks in favour of the time factor.

Of particular interest is the extent of disintegration obtained with different species of bacteria (Table VIII) since this gives a rough estimation of the resistance of the cells to disintegration in the X-presses. In this way, cocci (staphylo- and streptococci) and *Mycobacterium phlei* were found to be more resistant, which agrees well with the results obtained in studies on the killing effect of ultrasonic waves[23] and the thickness of the cell walls.[18] This indicates that the extent of the two methods of disintegration is influenced by some common feature, probably the mechanical strength of the cell wall. A more detailed investigation of this problem is planned.

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The X-press may be purchased from Biochemical Processes Inc., 250 Park Avenue, New York 17.

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Control of Stock Culture Preservation and Inoculum Build-up in Bacterial Fermentation

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Summary. Control of product quality throughout repeated fermentations depends upon maintenance of genetic uniformity from the time of strain selection until the product is harvested. Genetic control, therefore, must be maintained for stock cultures, and during inoculum preparation and growth to produce the product. Theoretical and applied aspects of a procedure that will accomplish this control are discussed. A specific procedure adaptable to situations in which a 'seed' is repeatedly increased to produce a larger quantity of a final material is described. The procedure was tested by comparing the product of three bacterial species grown by a standard or usual *vs.* recommended or experimental procedure comparing product quality and uniformity. Use of the recommended procedure resulted in the high production of a uniform product based on the maintenance of the colony type of *Bacillus anthracis*, colony colour and type of *Serratia marcescens*, and uniformity and amount of toxin produced by *Clostridium botulinum* type A.

One fundamental characteristic of living organisms is their capacity to vary genetically or phenotypically in all their morphological or physiological characteristics. Surprisingly little attention has been paid to the problem of controlling and increasing genetic stability. For any research, development or production purpose, although the volume of material fermented may vary, the inoculum volume of the desired strain must be increased repeatedly from a 'seed' to the final product that retains the unique characteristics desired. Repeated fermentation assumes that the technique has the ability to produce products: (1) in the required quantity, (2) of a minimum quality, (3) repeatedly and uniformly, and (4) economically (feasibly). For fermentations with these characteristics to be accomplished easily and routinely, it is essential that genetic variation is controlled, both to maintain product quality and to prevent interactions that are difficult to analyse and interpret.

From a review of the literature and from personal observations,

it appears that strains of micro-organisms are frequently transitory and many stock cultures are replaced because of uncontrolled genetic variation, not because of the development of a better strain. The abandonment of new product development because of 'change', which in reality is attributable to uncontrolled variation in the strain or inoculum during its build-up, must be very frequent. A review of the literature does not indicate that procedures to establish full genetic control of a population over long periods of time have been adequately published and proven. Possible exceptions to this conclusion are the methods used by Younathan and Barkulis[19] and Martin,[12] which appear to be scientifically sophisticated procedures used for their research or for pilot-plant work respectively. The simple and direct procedure of transferring the culture weekly from slant to slant, a procedure still widely used, has been proved repeatedly and expensively not to fulfil the minimum requirements of continuing experimentation, although presumably such procedures do meet the purposes of some experimentation. Considering the importance of the problem, it is surprising that this area is so frequently approached as an art rather than a science, particularly in the production of products like beer that are intended for direct human consumption.

The problem of maintaining quality throughout repeated fermentations is a much more difficult one than that of obtaining genetic stability. In various reports, workers have described control of one or more steps of the fermentation process in an attempt to maintain product quality and uniformity. The author has also used controls based on certain steps (such as stock culture maintenance), but whatever success is attained is temporary as some other step sooner or later varies. On the basis of this experience, an integrated process that maintains control of variability from stock culture to product harvest was developed.

It is the purpose of this report to discuss certain theoretical and applied considerations for strain maintenance and inoculum build-up and the use of simulated tests to evaluate quality. An example of a procedure incorporating these considerations is given. Several bacterial species were employed to evaluate the recommended procedure, and certain evaluation tests used throughout repeated fermentations with two species are reported.

I. Theoretical Considerations

In the development of a specific procedure, convenience of operation, the availability of equipment, the capabilities of the personnel, and the growth characteristics of the micro-organism require consideration. In addition, certain general considerations, as discussed below, must be recognized and met. As a consequence, procedures for different organisms or installations may differ somewhat in detail.

(1) *Basic information on physiology is required.* Certain minimum knowledge about the organism and fermentation systems is essential. Information is required on the colonial morphology and how it is affected by variation of the growth environment, and on the relationship of turbidity, pH, glucose utilization, CO_2 evolution, and other possible control information to the growth curve.

(2) *Stock cultures must be stabilized and used.* Numerous writers, for example Stanier *et al.*,[16] and Beesch and Shull,[2] point out the necessity of preserving strains to prevent genetic change. Procedures to freeze-dry fungi, yeasts and bacteria without change in observed characteristics have been presented.[3,14,17] Heckley and Klumpp[7] obtained high recovery following freeze-drying, and stabilization procedures that allow high survival during stabilization should be used.

(3) *Operating procedures and environmental conditions must be controlled.* Uniformity of the physical environment and the performance of techniques is required. Inadvertent or uncontrolled variation of environmental and physical factors may distort variability, and prevent its recognition. The steps of the process should be fully described and routine checks that each observation is within known statistical limits should be made. Instruments needed to control such variables as pH, incubation and storage temperature, aeration and medium constituent additions must be checked regularly for reliability. Records need to be kept of these variables. The medium ingredients required for control tests should be obtained in quantity so that only one lot of medium is used. The commonly used medium ingredients probably do not deteriorate during a 20–25-year period if full

bottles are stored (sealed and plastic coated) in a cool, dry, dark place. (Personal communication, Dr. Harriette D. Vera, Baltimore Biological Laboratories, Baltimore, Md.)

(4) *Genetic variation originates principally by mutation.* Colonial morphology and other readily observable characteristics are manifestations of physiological processes; consequently, consistency of the observable characteristics indicates genetic stability and, conversely, mutation or variation in the observable characteristics indicates genetic instability.[8,12] For the most part, bacteria are asexual and divide by fission; therefore, genetic characteristics, either mutant or non-mutant, will normally be continued in the clonal progeny. Variation originates principally by mutation, although other types of variation have been described.

The mutation rate per gene varies between a maximum of about 10^{-3} and a minimum that is, practically speaking, unobservable (i.e., above 10^{-10}). An average rate of non-lethal gene mutations will be considered as one mutation per 10^{8} gene duplications. In practice, a few specific mutations constitute the major portion of all mutations observed. Multiple mutations are so rare that they can be ignored; they occur at a rate that is the product of the respective mutation rates of gene A and gene B.

(5) *Only single colonies are used for developing stocks and inoculum.* Except for chain-forming species, colonies on semi-solid agar medium develop from single cells, as directly demonstrated by McNew[12a] and the probability of a single cell stock being developed multiplies with each successive plating of the selected colony. Even with chain-formers, and in most cases of multi-cell colony origin, the colony originates from clonal cells, 1, 2 or more generations removed from a parental cell. The possibility of two seeded loci growing together into one colony is minimized by seeding the plate with only a few cells.

Use of single colony stocks ensures the minimum chance of variation in the inoculum. A colony is a relatively small number of clonal cells, observed as an aggregate to be of the desired colonial characteristics. Since the stock cultures are preserved and maintained so that, as far as is known, mutation and selection are both minimized, the probability of the genetic uniformity of

each colony picked for inoculum remains constant throughout the time a stock culture is used.

The possibility of obtaining an unsatisfactory inoculum or product is further minimized by: (*a*) observation of progeny colonies for uniformity and acceptability at the end of the primary build-up of the inoculum and on the simulated final product; and (*b*) testing of the simulated product before the primary stock culture is accepted and used for inoculum.

This paper supports the pure culture rather than the mixed culture concept, a division noted by Beesch and Shull.[1]

(6) *Selection is the only force causing population shifts.* The incredible adaptability of a microbial population has been demonstrated in many ways, for resistance to chemicals,[15] with either increased or decreased virulence,[9] and for phage resistance.[11] Tables to indicate the relative importance of mutation and selection in effecting population shifts (see Wright,[18] for gametic selection), were presented by Lincoln,[10] who concluded that selection, operating upon a large heterogeneous population, is the only force that can effect wide and rapid shifts in the frequency of a gene. Virtually no microbial population is completely homogeneous and since variation for each gene or organism occurs at some fixed probability, the problem of genetic control is really one of minimizing selective growth. To this end, a high percentage of inoculum is used and, whenever possible, this inoculum is in the upper part of the log phase of growth. In addition, the final fermentation is stopped and processed as soon as, and as rapidly as possible after, product specifications are met. Lag time is minimized by always growing the culture in the final-type medium.

The need to use 5 to 10 per cent inoculum to prevent the lag phase (and to decrease fermentation time) is noted by Foster.[5] We have used inocula as high as 5 per cent. In this case, theoretically, about four cell generations are required to produce inoculum stopped at the peak of log growth, and about 4.3 generations are required for the final fermentation, as selective growth is most evident during these periods. After fermentation is completed, possible selective growth is prevented by refrigeration or freezing, by the addition of bacteriostatic materials, and, with aerobes, by stopping aeration.

(7) *Simulated tests have a high prediction value.* Simulated tests are highly predictive for product evaluation. If this were not true, scale-up of a laboratory observation would be most difficult and indirect; Gaden [6] noted that the general practices of chemical process scale-up are applicable to microbial fermentation by isolating the basic variables.

(8) *Decisions are based on specifications.* The specifications set on the several tests must be realistic and discriminatory. The minimum standard set, preferably from experimental observations, should be conservative enough to guarantee that any fermentation developed from the stock culture or from the build-up inoculum will produce an acceptable fermentation more than 99 per cent of the time. Specifications are established as a basis for decision, and the only decision to be made regarding a stock culture or an inoculum is that it is acceptable or unacceptable. Failure to meet any one of several specifications causes rejection.

II. General Outline and Basis of the Procedure

The general outline of the procedure is given in Fig. 1. The selected strain is preserved in numerous genetically uniform subsamples, each of which becomes a proven stock culture after specified tests of acceptance to prove that minimum quality is met. These stocks are stored under conditions which ensure maximum longevity. At definite time intervals a proven stock culture is plated, and from the resulting population of colonies a single colony is selected and 'built up' to produce an 'unproven' inoculum. The quality of the unproven inoculum is subsequently proven by appropriate tests, including a simulated product test. If all test results prove the inoculum acceptable, it is used for a designated time as inoculum for the fermentation.

III. Specific Recommended Procedure

(1) *Selection of the strain.* The potentially useful strains are screened and the best strain selected with regard to the properties under consideration. Several single-colony isolates of the stock

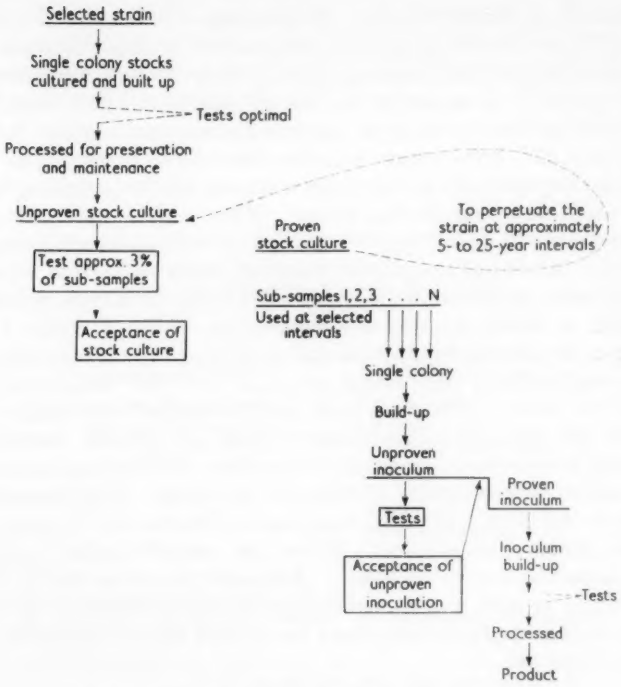

Fig. 1. General scheme for seed stock maintenance, proving and build-up

should be grown and the desired characteristics verified in the sub-isolate. This sub-stock is then preserved for stock culture use.

(2) *Preservation of a stock culture.* The detailed procedure for producing a stock culture of the selected strain is given in Fig. 2. A minimum of ten 250-ml flasks, each containing 20 ml of medium, are each seeded with a single colony picked from surface plates and observed at intervals for initiation of turbidity. This observation of turbidity relates the stage of growth to a known point on the standard curve. Growth is continued to the estimated maximum for the log phase as determined from turbidity, pH, sugar

utilization, or similar curves. At this time the culture is transferred, 1 ml into 19 ml of fresh medium (or in larger increments maintaining this ratio), and again grown under standard conditions until growth is a maximum for the log phase, at which time the culture is used as inoculum to produce the required amount in the ratio of 1:20. This culture is grown until the stationary phase of growth, because cells at this stage are more stable to drying than those collected from the log phase. Without delay, the cells are concentrated by centrifugation and resuspended in 60 ml of supernatant. An equal volume of stabilizer, such as bovine serum, 20 per cent glycerine, 10 per cent skim milk, or Naylor-Smith[13] diluent is added to the cell suspension. After mixing, 1-ml samples are dispensed into vials and a viable cell count determined. The suspension is shell frozen at $-20°$ to $-70°$ and stored at $-20°$ or lower while three tests are conducted: (1) viable cell count per ml; (2) contamination; and (3) genetic variation. Storage at this stage is limited to three days, and drying is usually completed before results of tests are available. The procedure used at the Northern Utilization Branch, Peoria, Ill.[17] is used for stock culture preservation. Tubes are cut off under vacuum, and stored at $-20°$C or lower. Although our experience in this respect is limited, experience at Peoria and elsewhere indicates that stock cultures are stabilized for at least five and probably for 20 to 25 years.

Surface plate, observe colonies and pick desired type of single colonies

Build up to volume required for the sub-samples (see text)

Concentrate 20 times; add serum

Freeze at $-20°$C

Test. Determine:
- (*a*) viable cell count/ml
- (*b*) contamination
- (*c*) genetic variation

Accept or reject the lot, discard rejected sample

Distribute desired volume into desired number of sub-samples

Process by preservation method (drying)

Identify and designate lots as unproven stock culture

Fig. 2. Detailed procedure for producing a stock culture of the selected strain

(3) *Proving of the stock culture.* As indicated in Fig. 3, in proving the stock culture, at least three per cent of the dried sub-samples are assayed in tests (described below) designed to prove the quality of the seed stock.

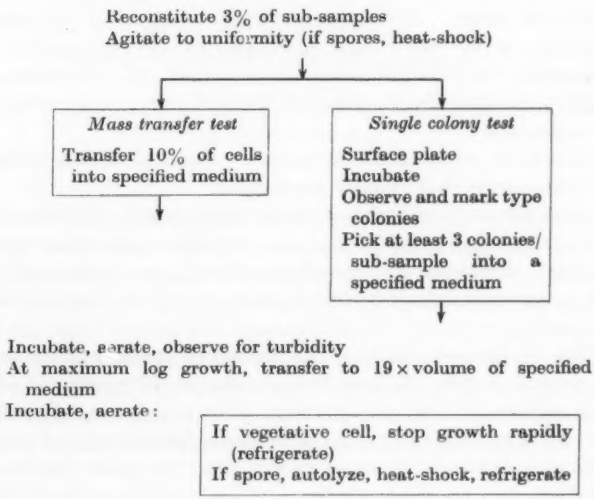
Reconstitute 3% of sub-samples
Agitate to uniformity (if spores, heat-shock)
Mass transfer test
Transfer 10% of cells into specified medium
Single colony test
Surface plate
Incubate
Observe and mark type colonies
Pick at least 3 colonies/ sub-sample into a specified medium
Incubate, aerate, observe for turbidity
At maximum log growth, transfer to 19 × volume of specified medium
Incubate, aerate:
If vegetative cell, stop growth rapidly (refrigerate)
If spore, autolyze, heat-shock, refrigerate
Test. Determine:
1. Viable cell count
2. Storage stability, accelerated test
3. Contamination
4. Genetic variation
5. Miscellaneous tests, as appropriate
a. Determine whether each test meets minimum specifications
b. Discard stocks not meeting ALL specifications
c. Identify each sub-sample of the seed stock meeting ALL test specifications
Accept and identify one stock as proven stock culture

Fig. 3. Detailed procedure used to produce a proven stock from an unproven stock

(*a*) Viable cell counts are made immediately after drying, and the percentage recovery is calculated from this count and the previously obtained initial cell count. The percentage recovery should be high, i.e. above 70 per cent, in order that selective death due to freezing, drying, or reconstitution may be minimized. The count taken after freezing is used for information on comparative loss due to freezing and drying.

(*b*) The loss of viable cells in the dried stock should not exceed 5 per cent per year. Storage loss can be minimized by storage at $-20°C$ or lower. Accelerated storage tests, after the pattern of Fernelius, *et al.*[4] have been developed for several species, but only research extending through many years will determine the correlation between the accelerated test and growth characteristics of the organism.

(*c*) Allowable contamination is zero. Contamination indicates faulty techniques that require correction.

(*d*) Five per cent colonial or observable genetic variation may be tolerated. Observable genetic variation indicates genetic instability; therefore, all variations should be minimized even though later tests will, if discriminatory, eliminate variant stocks before use.

(*e*) The viable cell count of the preserved stock culture should equal approximately the number of cells per ml produced in the mature culture. This requirement allows the use of uniform procedures when different strains are being produced and guarantees enough colonies when surface plated to grant freedom in comparing and choosing colonies.

(*f*) Still other tests (such as toxin or antigen production, virulence, or fermentative properties) should be specified if critical and differentiating tests are known.

(*g*) The stock culture is accepted if the minimum standards set for each of the tests are met. Each sub-sample is identified and designated as a proven lot. If any of the tests are not met, the entire lot is rejected and destroyed.

(4) *Production of inoculum.* To prepare an inoculum, for which procedures are given in Fig. 4, one sub-sample of the proven seed stock is used to prepare inoculum for a designated succeeding interval of time. In some cases we have used four months as the

Reconstitute sub-sample, surface plate, incubate
Observe and select single colonies
Pick one colony into specified medium, repeat x times
Incubate; aerate
At maximum log phase, transfer into 19 × volume of medium
Incubate, aerate:
A. Repeat until desired number and volume of inoculum produced
B. For vegetative cells, refrigerate;
for spores, autolyze, heat-shock, refrigerate
Freeze at −20°C, store at −20°C (or lower)
Identify and designate lots as unproven inoculum

Fig. 4. Detailed procedure used to produce an unproven inoculum from a proven stock culture

interval. The sub-sample of the proven stock culture is surface plated, and, after colony observation, single colonies are picked for growth by procedures similar to those given in Section 2 above. To furnish one inoculum sub-culture per week through four months, only 400 ml of culture grown just to the stationary phase are needed. The bulk inoculum immediately after its production is dispensed in 20-ml amounts in test tubes, plug frozen at −20°C or lower, and stored at −20°C or lower. Glycerine or other additives may be added prior to freezing, if desired.

(5) *Proving of the inoculum.* Procedures to prove the acceptance of an inoculum are given in Fig. 5. When a tube of the inoculum sub-sample is selected for use or test, it is thawed by placing the tube in tap water and shaking it gently until the ice is melted. Experiments (unpublished) have shown that as long as ice is present in the tube, the rate of thawing (temperature of the water bath) is unimportant. At least 3 per cent of the inoculum sub-cultures should be tested prior to acceptance of the inoculum. The tests are straightforward checks to determine that the viable cell count equals at least 80 per cent of that of a full-grown culture, that contaminants are absent, and that colonial variation is less than 0·1 per cent; other tests are made as appropriate.

For many fermentations the simulated tests and the acceptance

Thaw 3% of tubes with gentle shaking at 15°C

Test. Determine:

1. Viable cell count
2. Contamination
3. Colonial variation
4. Miscellaneous tests, including product as appropriate
5. Simulated product test
   a. 1st transfer
      (1) transfer 5% of cells to specified medium
      (2) incubate, aerate, grow to maximum log phase of growth
   b. 2nd transfer. Repeat 5a (1) and (2) above
   c. 3rd transfer. Repeat 5a (1) and (2) above
   d. 4th transfer. Repeat 5a (1) and (2) above
   e. 5th transfer—repeat 5a (1) and (2) above except grow to final concentration for product production. Final volume to be great enough for all tests, grow to final cell and product concentration. (1) Conduct tests 1, 2, 3, and 4 above.

Determine whether each test meets minimum specifications

Discard proven seed stocks and unproven inoculum of selection not meeting all specifications

Designate, identify and date sub-samples of all unproven inoculum that meets all specifications as

Proven Inoculum

Fig. 5. Detailed procedure used to produce a proven inoculum from an unproven inoculum

tests will be identical. No attempt is made to force the laboratory to duplicate pilot or production conditions, as this is only possible to a certain extent. However, it is important that the fermentation medium and other readily duplicated variables should be incorporated into this test. Fig. 5 shows a series of five successive transfers proposed for the simulated test. More cell generations should be grown than will occur in the final fermentation, because the test becomes more discriminatory as more generations are tested. Greater discrimination is also possible with lag or stationary phases of growth. At the conclusion of the simulated test, the viable cell count and product evaluation should meet the specifications, and genetic variation should be less than 1·0 per cent (based on the observation of at least 3,000 colonies). Lots meeting standards for all tests are designated proven inoculum; those failing acceptance are rejected and destroyed.

(6) *Production of the final fermentation.* The final product is produced by procedures detailed in Fig. 6. One sub-sample of the proven inoculum is used to initiate each batch of final fermentation product. No proven inoculum is older than the designated interval for replacement plus the lead time required to complete acceptance tests. Sub-samples are not subjected to repeated freezing or thawing.

Thaw one sub-sample stored at $-20°C$ (or lower) by gentle agitation in cold water.

Transfer to specified medium using inoculum at ratio of 1:20. Run control measurements as desired.

Transfer in maximum log growth phase to final volume. Run control measurements as desired.

At maturity, cool rapidly. Process without delay to produce final product.

Final Product

Fig. 6. Detailed procedure used to produce the product starting with a proven inoculum

(7) *Maintenance of product uniformity.* The possibility of an unsatisfactory final fermentation is minimized by maintenance of a tested stable stock culture in a manner that minimizes mutation or selection. This procedure maintains throughout the entire time of using the stock culture the same probability for each colony originating from a single cell. Colonies selected are observed, to verify that they are of the desired type, not mutants, and do not contain a mutant sector. Progeny populations of each selected colony are observed at two different times; (1) at the end of the inoculum build-up; and (2) at the termination of simulated product test. Observation of significant numbers of mutant colonies, i.e. 1 per cent, at either stage would disqualify that stock for use. The quality of the fermentation is tested by a simulated test before the inoculum is used; this test can be made more or less discriminatory as desired.

Uniformity of repeated fermentations is assured by: (1) use of a stabilized stock culture; (2) testing and verifying the performance of the stock culture in simulated use; and (3) the use of pretested inoculum that meets critical specifications.

In addition, experience has shown that uniformity is increased after a definite procedure is specified and used, presumably because of more operational familiarity and confidence, and the unconscious elimination or standardization of some of the unrecognized or minor variabilities.

It is to be noted that completion of the designated tests requires several days; consequently, a lead time is required. In practice, each time single colonies are selected, although only one colony is ultimately used, several colonies will be picked and carried through all the tests as an insurance for having at least one colony acceptable. In working with the species enumerated later in this report, relatively few colonies are discarded, and picking three to five colonies virtually guarantees at least one acceptable stock or inoculum; however, as a matter of policy it is desirable to produce and maintain several acceptable stocks of inocula.

Although we have not observed the phenomena, trends indicated in regressional analysis would presumably denote change in and the need to replace seed stocks.

(8) *Comparison of usual and recommended procedures.* Several characteristics of the recommended procedure are contrasted with the corresponding characteristics of some standard procedures as follows:

Prevalent (standard procedures)	Recommended procedure
1. Stock cultures are maintained by serial transfer, generally of vegetative organisms	1a. Stock cultures are maintained in stable form without transfers except at regular intervals expected to be once in 5 to 25 yrs
2. Genetic variation is uncontrolled or control attempted only after loss of desired characteristics is experienced	2a. Genetic variation is controlled in: (1) the stock culture, (2) the proven stock culture, and (3) the proven inoculum
3. Mass transfer of many colonies or the washing of slants is used for stock culture or inoculum production	3a. Only single-colony isolates are used
4. Physiological age of inoculum is disregarded	4a. Inoculum is transferred in log phase (if possible)
5. Cells are grown on solid medium and/or media not necessarily similar to the medium used for fermentation	5a. Except to observe colonial variation and to pick single colonies, the medium used to grow cells is that used in production

continued

Prevalent (standard procedures)	Recommended procedure
6. Amount of inoculum varies widely	6a. Five per cent inoculum is used
7. Inoculum is transferred at a convenient time	7a. Inoculum is transferred at the highest population in the log phase
8. Number of cell generations to produce a product is not controlled	8a. The same average number of cell generations occur in every fermentation
9. Discriminatory tests are not made except on the final product	9a. Discriminatory tests are made prior to acceptance of: (1) the proven stock culture, and (2) the proven inoculum. Less discriminatory tests are made while developing the unproven seed stock and the final product

IV. Experimental Results

The procedure reported in this paper was compared with a standard procedure by evaluating characteristics of the products of the two procedures. The standard procedure given for each organism is the one that was already in use with that species. In all cases, the fermentation medium composition, pH, growth temperature and aeration were the same for testing the two procedures. The composition of the bacterial population was determined by observing 1,500 to 2,000 colonies grown by a surface-plate technique for colonial characteristics or, in the case of toxin, by titration with mice.

(A) Clostridium botulinum *type A*; *characteristic tested—toxin production.* In the standard procedure a spore stock of the Hall strain was maintained in dry soil, and re-entered as a source of inoculum whenever the stock in use became atoxigenic (about four times a year). Stocks used from day to day were maintained in defatted meat cubes. Toxin for both comparisons was produced in an enzymatic digest of protein.

The strain used to test the recommended procedure was selected after a survey of 262 single colonies for toxin production and other growth characteristics. Spore stocks of the proven seed stock

were prepared in trypticase soy medium plus 0·5 per cent agar and used for the day to day inoculations.

In a series of 26 consecutive fermentations, the toxin production by the recommended procedure was 1,450,000 ± 98,000 mouse LD_{50} per ml while the standard procedure produced 770,000 ± 282,000 mouse LD_{50} per ml.

(B) Bacillus anthracis; *characteristic tested—colony morphology.* The standard procedure maintained spore stock cultures in the lyophilized state. A sample of these spores was resuspended in water, heat-shocked, and used to inoculate nutrient agar slants. After sporulation the growth was washed off in water, heat-shocked, and used as a bulk seed inoculation. Portions of the bulk seed were used to inoculate Erlenmeyer flasks. These cultures after sporulation were heat-shocked and used for virulence tests. On three occasions (as noted in Table I), two further successive transfers were made.

Table I. Percentage of desired and mutant colonies observed in the product comparing two methods of seed culture and inoculum build-up

	Standard procedure			Recommended procedure		
	No. of samples	Desired, %	Variant, %	No. of samples	Desired, %	Variant, %
Lyophilized stock culture	20	100	0	12	100	0
Bulk stock culture	5	94	6	21	100	trace
Inoculum flasks	21	69	31	40	99·8	0·2
2 transfers beyond seed flask (1 sporulation)	3	6	94	3	99·0	1·0

The seed stock for the recommended procedure was a single-colony stock selected for high virulence after screening 15 colonies. The fermentation medium was a peptone–yeast extract type for both comparisons. The comparison of colony production by a standard method and the method recommended in this paper is given in Table I. The evidence is overwhelming proof of the value of single-colony seeding and inoculum control. Zelle *et al.*[20]

show the relationship between colony morphology and virulence of this strain, and this summary is another treatment of data collected during that work.

(C) Serratia marcescens, *characteristic tested—colony morphology and colour*. The production of a smooth, dark-maroon colony type of *Serratia marcescens* was stabilized following use of the recommended procedure. The use of this procedure was preceded by periods when it was not possible to produce uniformly pigmented cultures. During these periods, stock cultures were maintained by serial transfer at 6 to 8 week intervals on Wilson's peptone agar slants.

In the first attempt to correct this difficulty, a smooth, dark maroon colony that pigmented at 18 h was isolated from the mixture of types in the stock, most of which produced a pink pigment following 30 h of growth. The early-pigmenting type was found to be stable and uniform with regard to pigment production and colony type after several serial transfers on Wilson's peptone agar medium. Slants were used as seed stocks, but after 18 months, the problem of colonial variation again occurred. An effort was then made (Dr. T. L. Snyder and Mr. H. Lee, unpublished) to select a single-colony strain that was genetically stable and to define the optimum growth conditions to produce cells or maintain stock cultures and to prevent selective growth.

To obtain an isolate of maximum stability, 113 single-colony stocks were picked from five sub-cultures of the dark maroon colony and transferred serially for seven successive times at 48-h intervals on nutrient agar. Two stocks remained more than 80 per cent pigmented. These two stocks and seven others were compared for stability after seven successive transfers at 24 and 48-h intervals. Only five strains showed a high degree of stability in the 24-h transfer procedure and, of these, only one had a relatively high stability in the 48-h transfer series.

Other experiments with the selected stock showed selection was less severe when the growth temperature was 12°C rather than a higher or lower one, when transfers were made in broth rather than on agar slants, and that maintenance of the pH of the culture below 7·8 had a significantly favourable effect on maintaining stability. A single-colony isolate was made and used as a stock culture.

After the reselected stock was proven and for approximately 4

years while it was grown by the recommended procedure, no further difficulty was experienced in maintaining uniform pigmentation and colonial morphology. The cultures produced by this procedure contained more than 99·5 per cent of the desired colonial type.

V. Discussion

The technique described in this paper for stock culture maintenance and inoculum build-up has, in our hands, produced satisfactory cultures repeatedly and uniformly. Actually, the important thing about this procedure is the integration of facts and a philosophy of operation into a procedure. The facts are obtained in some cases experimentally, in others inductively. The correctness of the philosophy and completeness of the facts are tested by practice. At times it is necessary to modify the ideal procedure in recognition of the limitations of equipment, time schedules, and personnel qualifications. The recommended procedure is applicable, with slight modifications, to any type of production involving build-up from a small 'seed' to a larger quantity of identical material. Our procedure was developed for vegetative aerobic bacteria, but only slight modifications would be required to adapt it for anaerobes, spore-formers, tissue culture cells, viruses or fungi. The test can be easily increased or decreased in sensitivity by changing the test specifications or by specifying that fewer or more cell generations or culture transfers be observed in the simulated product test.

One may conclude that the effort spent in maintaining seed stocks and providing inoculum by the procedure recommended in this paper is time-consuming and expensive. It is. The alternative has been eventual loss of the stock, preceded by a period of variable and irregular production. Unless the stock on which research is done is maintained unchanged, all information on its production becomes questionable and must be verified again on another alternate strain. Frequently, procedures need to be modified to use the replacement strain. The loss of stock cultures and variability of fermentation is expensive, and the occurrence and amount of loss is unpredictable. It is believed that a definite scheduled programme of stock culture maintenance and inoculum testing is both preferable and less expensive.

Some may consider the selection and use of single-colony stocks as risky; however most, if not all, evidence available indicates that fermentations using the best pure cultures are superior to those using mixed cultures. In this recommendation the repeated observation and testing, particularly the simulated product test, guarantee with a high predictability that quality will be obtained. In our experience, the procedure results in uniform, repeated production at or near the maximum that the strain, medium, and general conditions allow.

VI. Summary

A procedure that maintains genetic control, minimizes selective or adaptive growth, and evaluates with discriminatory tests the value of the product is presented for seed stock maintenance, inoculum build-up, and simulated product. The reason for using these procedures is discussed in a consideration of the theoretical and practical factors that must be controlled or accepted. The specific procedure is an attempt to develop a philosophically and theoretically defendable operation that integrates numerous uncoordinated observations and accepts the practical limitations of laboratory equipment and personnel.

The specific procedure has been tested with three different bacterial species with readily tested genetic characteristics. The tests demonstrate that genetic stability and product uniformity can be guaranteed with high probability by use of this procedure.

Acknowledgments. I am indebted to numerous colleagues who have contributed toward the final development and testing of the procedure and concepts discussed in this paper, particularly Mr. G. B. Achorn, Jr., who supported this area of work with enthusiasm, Dr. M. R. Zelle, who initially demonstrated at these laboratories the dramatic possibilities of applied genetics, Dr. T. L. Snyder and Mr. Hugh Lee for use of their data as indicated in the text, and Mr. G. W. Schmersahl and Dr. P. R. Middaugh for determining the effect of freezing on the stability of inoculum.

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